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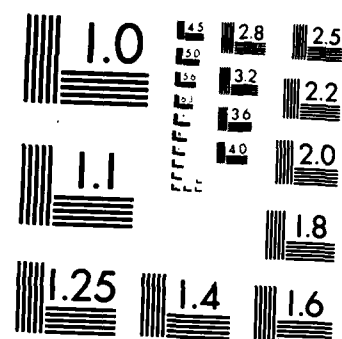
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**The Interaction of Coagulation Factor V and Vascular Endothelial Cells**

**a Thesis by Thelma Jannette Cervený, Major, USAF, 124 pages**

**1984**

**Doctor of Philosophy degree awarded by**

**the University of Minnesota, Mayo Graduate School of Medicine**

**Mayo Clinic/Foundation**

**ABSTRACT**

Bovine aortic endothelium was studied *in vitro* with respect to synthesis of coagulation factor V. Supernatant samples and solubilized cells, from confluent cultures, were analyzed for factor V in a two-stage bioassay and in a double-antibody radioimmunoassay. Confluent cultures were processed for indirect immunofluorescence using monoclonal and polyclonal primary antibodies. Preconfluent cells were pulsed with  $^{35}\text{S}$ -methionine in methionine-free culture medium and resultant culture supernatants were chromatographed on factor V monoclonal antibody-Sepharose resin to isolate the intrinsically labelled  $^{35}\text{S}$ -factor V. Isolated materials and  $^{125}\text{I}$ -factor V/Va standards were analyzed by electrophoresis followed by autoradiography.

The bioassay revealed an increase of functional factor V, with time, in the culture supernatant, while solubilized cells were negative for functional factor V. Radioimmunoassay results indicated an increase, with time, of factor V/Va antigen in culture supernatants, and a constant level of antigen per cell from solubilized cells. Not all cells provided a positive fluorescence signal, endothelial cells stained differentially for factor V/Va with both the monoclonal and

polyclonal antibodies. Autoradiograms of electrophoresis gels with samples from immunoadsorbed  $^{35}\text{S}$ -culture products, and  $^{125}\text{I}$ -factor V/Va standards revealed labelled proteins with electrophoretic mobilities consistent with  $^{125}\text{I}$ -factor V/Va standards. Data from bioassay, radioimmunoassay and  $^{35}\text{S}$ -methionine incorporation each indicated factor V had been synthesized by cultured bovine aortic endothelium, while immunofluorescence data indicated factor V was associated with endothelial cells.

Additional, preliminary studies, associating factor V and the prothrombinase complex with endothelium, were accomplished through  $^{125}\text{I}$ -factor V/Va binding and S-2238 chromogenic assays.  $^{125}\text{I}$ -factor V/Va binding indicated factor V/Va definitely binds to endothelial cells *in vitro*. And S-2238 assays indicated that endothelial cells can provide a surface/receptor site for prothrombinase complex formation.

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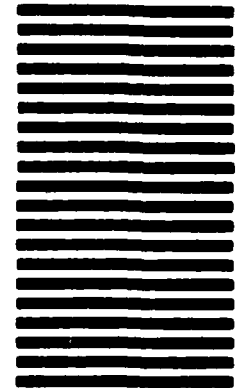
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VASCULAR ENDOTHELIAL CELLS

A THESIS

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL  
OF THE UNIVERSITY OF MINNESOTA

By

THELMA JANNETTE CERVENY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

JUNE 1984

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## ABBREVIATIONS USED

ATIII - anti-thrombin III

BAE - bovine aortic endothelium

BSA - bovine serum albumin

C-3 - Cytodex 3 microcarriers

Ca<sup>++</sup> - calcium, present as calcium chloride

CNBr - cyanogen bromide

DAPA - dansyl- argininepiperidineamide

ECGS - endothelial growth supplement, used in human endothelial cultures

Factor II - prothrombin

Factor IIa - thrombin

Factor VIII: Ag - factor VIII: antigen

Factor VIII: vWF - factor VIII: von Willebrand factor

FBS - fetal bovine serum

FITC - fluorescein isothiocyanate

g - gravity, referring to centrifuge spin force

gm - gram

HEPES - N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IgG - immunoglobulin G

L - liter

M - molar

mCi - millicurie, 10<sup>-3</sup> Curie

mg - milligram, 10<sup>-3</sup> gram

ml - milliliter, 10<sup>-3</sup> liter

mM - millimolar, 10<sup>-3</sup> molar

ug - microgram,  $10^{-6}$  gram

ul - microliter,  $10^{-6}$  liter

uM - micromolar,  $10^{-6}$  molar

ng - nanogram,  $10^{-9}$  gram

PBS - 0.01 M phosphate buffered saline

PCPS - phosphatidyl choline phosphatidyl serine

PEG - polyethylene glycol

S-2238 - H-D-Phe-Pip-Arg-NH-  $\text{-NO}_2 \cdot 2\text{HCl}$ , an artificial thrombin substrate

SDS - sodium dodecylsulfate

TAGIT - N-succinimidyl-3-(4-hydroxyphenyl)-propionate

TEMED - N,N,N,N-tetramethylethylene diamine

U - units, used in reference to thrombin activity

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## INTRODUCTION

Endothelium is a widely dispersed "organ" of diverse capabilities. It provides the lining for the cardiovascular and lymphatic systems. While this lining is ubiquitous, it is not all one piece, the individual cells are assembled together to form a mosaic. Histologically, endothelial cells from different vascular sites display various individual morphology (1,2). Studies of endothelial ultrastructure have revealed regional differences with respect to organelles and intercellular connections (3), and biochemical techniques have exposed even greater specialization at the molecular level (4). Recently, as awareness of the complexity of endothelium has increased, the realization that it is not simply an inert barrier between blood and tissue has dawned. The endothelium is a widely distributed organ of great biological potential, that not only extends throughout the body providing a non-thrombogenic vascular lining, but is intimately involved in other distinctive biological functions in various vascular sites and in particular organs (1).

Regional differences in biological function of endothelium are most apparent when comparing arterial and capillary walls: the capillary wall is virtually only endothelium, while arterial endothelium is the innermost of a complex number of layers consisting mainly of muscle and connective tissue. Arterial endothelium also differs from venous endothelium in structure and function. It seems almost every organ has endothelium adapted to its needs.

It is unlikely that the properties endothelium has during fetal and new born stages persist throughout the life of the organism. As with other organs and tissues, changes probably occur in response to environmental and physiological stimuli. It would be reasonable to assume that endothelium, because of its



location as the lining of the vasculature, would continuously adapt to the changes in blood composition, volume of flow, and pulsatile pressures, which continuously operate over a lifetime.

The vascular endothelium represents a unique case of epithelial differentiation, that makes possible massive exchanges between the blood plasma and interstitial fluid. Other functions of endothelium include macromolecular synthesis of proteoglycans, the production of proteases, interaction with other cell types -i.e. leukocytes and platelets, regulation of blood flow, control of inflammatory reactions, and control of blood coagulation and clot removal (1,2,5).

Traditionally three factors have been considered important in the phenomena of thrombosis and hemostasis: the interaction of plasma procoagulants, stasis of blood flow, and the contributions of platelets and vascular endothelium (1,2,6). Many researchers have suggested that thrombosis starts at the level of the vascular wall. Injury of the vascular endothelium exposes basal lamina fibronectin to blood, and this rapidly leads to platelet adhesion and aggregation, as well as activation of the blood's soluble phase clotting system (2). These phenomena lead to the development, and growth, of a hemostatic plug, or mural thrombus, on and around the injured endothelium and exposed subendothelial layers.

A new interest has recently developed in the study of vascular endothelium. The development of tissue culture techniques pioneered by Jaffe et.al. (7,8) and others have made possible study of endothelial cells in vitro. Endothelial cells can now be removed and isolated from their basal lamina and successfully grown in culture. This recent interest in the study of endothelial

cells has provided a new source of information, indicating that endothelium is directly involved in certain aspects of thrombosis and hemostasis. It has become accepted, that endothelial cell function can be influenced by substances which also influence certain aspects of blood coagulation (9,10). Recent studies indicate that endothelial cells are responsible for synthesis, storage, and even release of substances which directly affect blood coagulation mechanisms (2). Endothelium is now being viewed as having a direct role in blood coagulation reactions (2).

The hemostatic blood clotting response to trauma occurs in the region of damage, and does not consume the total systemic coagulation-related humoral and cellular elements in blood. The response, and regulation, of this system is most likely a result of accelerated production of clotting activity by coagulation factor complexes in the local trauma area, which are prevented from disseminating from the site by downstream clotting inhibitors. When the response starts, formation of coagulation factor complexes provide a rapid delivery of enzymatic activity to the trauma area. This complex formation enables enzyme localization via the interaction of cellular and humoral components of the blood coagulation system.

The enzymatic complexes, generated by the humoral components of the coagulation system, are depicted in Figure 1, from Mann and Fass (11). The intricate feedback activation mechanisms of the contact activation system results in the initiation of "intrinsic blood coagulation" and factor XI activation. This first enzymatic complex consists of factor XII (Hageman factor), prekallikrein, high molecular weight kininogen, factor XI and an ill-defined negatively charged "surface", which provides a reaction site. The consequence of this

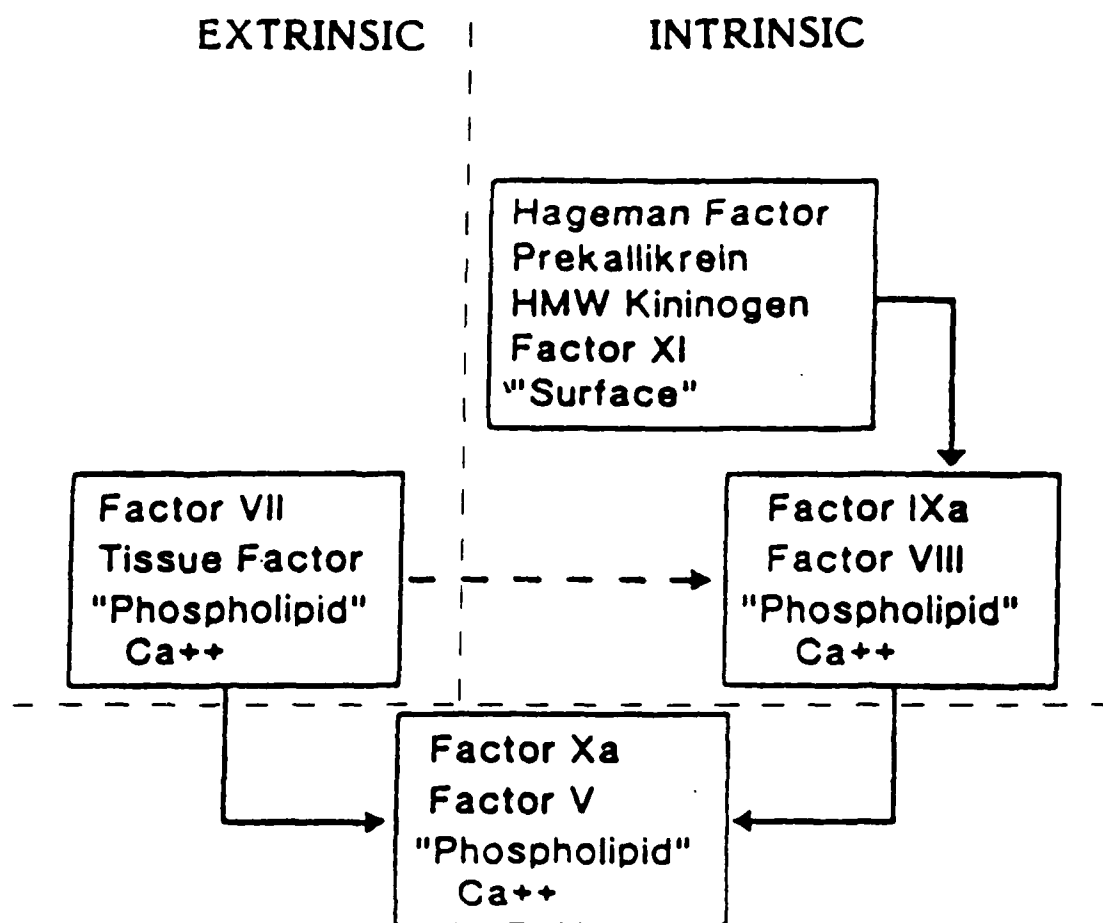


Figure 1

Figure 1. The enzymatic complexes of the blood coagulation system. The substrate of the first complex of the intrinsic portion of the cascade is factor XI, which, by an intricate cooperative feedback mechanism with the other components of the complex, becomes converted to factor XIa, which in turn activates factor IX to IXa. Factor IXa then plays an integral role in the next complex, catalyzing the conversion of factor X to Xa. The extrinsic pathway's initiating complex has two substrates, one is factor IX, which is converted to factor IXa, and the other is factor X, which is converted to Xa. The last complex, whose enzyme element is factor Xa, is the common point upon which both the extrinsic and intrinsic pathways converge. This complex, called "prothrombinase", catalyzes the conversion of prothrombin (factor II) to thrombin (factor IIa).

intricate activation results in enzymatic complex activation of factor XI to XIa, which in turn activates factor IX to IXa. The next complex down the cascade consists of factor IXa, the cofactor VIII(a), calcium, and a phospholipid surface. The substrate of this complex is factor X, which becomes activated to factor Xa. Factor Xa then becomes an integral part of the next enzyme complex with cofactor V(a), calcium, and, again, the phospholipid surface. This last enzyme complex is known as the prothrombinase complex (11,14-17). Prothrombin, factor II, is the important zymogen which is catalyzed to thrombin (IIa), by activity of the prothrombinase complex. Thrombin then cleaves fibrinogen, its most abundant substrate, to yield fibrin polymer, which becomes cross-linked/stabilized by the action of activated factor XIII (XIIIa), forming a net-like skeleton which entraps platelets and other cells and debris to form a clot. Thrombin also activates "feedback" substrates, such as factors V and VIII, as well as the fibrin cross-linker, factor XIII, and its own zymogen prothrombin.

Prothrombinase has been the most extensively studied blood clotting complex. The availability of purified complex components has enabled studies to provide a reasonable description of how the complex is assembled, and why this assembly is so crucial to timely regulation of hemostatic crises (11). The significance of complex formation, with all elements present, is demonstrated in Table 1. This table represents the relative importance of complex components in enhancing prothrombin conversion by the enzyme factor Xa, as compared to factor Xa alone, which has a relative rate of conversion of 1. These relative rates illustrate the significance of having all complex components present for optimum prothrombin conversion. Deletion of any one

Table 1. Relative Reaction Rates of Components of the Prothrombinase Complex in Various Combinations of the Components.

<u>Components present</u>	<u>Relative rate</u>
Xa	1.0
Xa, PCPS	1.0
Xa, Ca <sup>++</sup>	2.3
Xa, Ca <sup>++</sup> , PCPS	22.0
Xa, Ca <sup>++</sup> , PCPS, Va	278,000.0
Xa, Ca <sup>++</sup> , Va	356.0

Table 1. Relative reaction rates of components of the prothrombinase complex in various combinations of the components. Rates are all expressed relative to the enzymatic action of factor Xa alone. PCPS = phosphatidylcholine-phosphatidylserine vesicles, an artificial phospholipid surface upon which optimum prothrombin conversion to thrombin occurs in vitro.  $\text{Ca}^{++}$  = calcium present as  $\text{CaCl}_2$  in the reaction mixture. Va = factor V which has been activated by thrombin to factor Va the active cofactor in the prothrombinase complex.

component decreases the reaction rate at least 1000-fold. The interactions of the enzyme (factor Xa) with its cofactors (factor Va,  $\text{Ca}^{2+}$ , phospholipid surface) have been studied using phosphatidyl choline phosphatidyl serine (PCPS) vesicles as a model surface, see Table 1. In addition, the molecular interactions of factor Xa and factor Va have been accomplished (51), with the platelet serving as the phospholipid surface. These studies indicate that factor Va seems to be the platelet or phospholipid-bound receptor for factor Xa, and that factor Va is responsible for localizing factor Xa onto cell or phospholipid surfaces (11,15,17,43,51). While the PCPS-prothrombinase model, used to obtain the information in Table 1, cannot totally reflect the complexity of cell systems, eg., platelets, it can provide a workable model for prothrombinase function in vivo (14).

A hypothetical structural model of the microenvironment in which prothrombin catalysis takes place was proposed by Nesheim et al. (15), see Figure 2. The curved surface represents a phospholipid vesicle surface. The hydrophobic factor V molecule is embedded in the surface with factor Xa bound in a 1:1 stoichiometry. The cleavage of factor V to factor Va, is depicted by the large cleft in the model, within which the catalytic element of prothrombinase (factor Xa) can be sequestered with prothrombin, thus greatly enhancing the enzymatic reaction rate. It is likely that a similar process occurs on the platelet surface, as binding studies of factors Xa and Va on platelets also indicate a 1:1 stoichiometry (51). Prothrombin is shown localized within the Va-Xa complex by  $\text{Ca}^{2+}$  bridging to the phospholipid surface.

Platelets play an important role in maintaining hemostasis, and may provide a surface for formation of the prothrombinase complex in vivo (43,51).



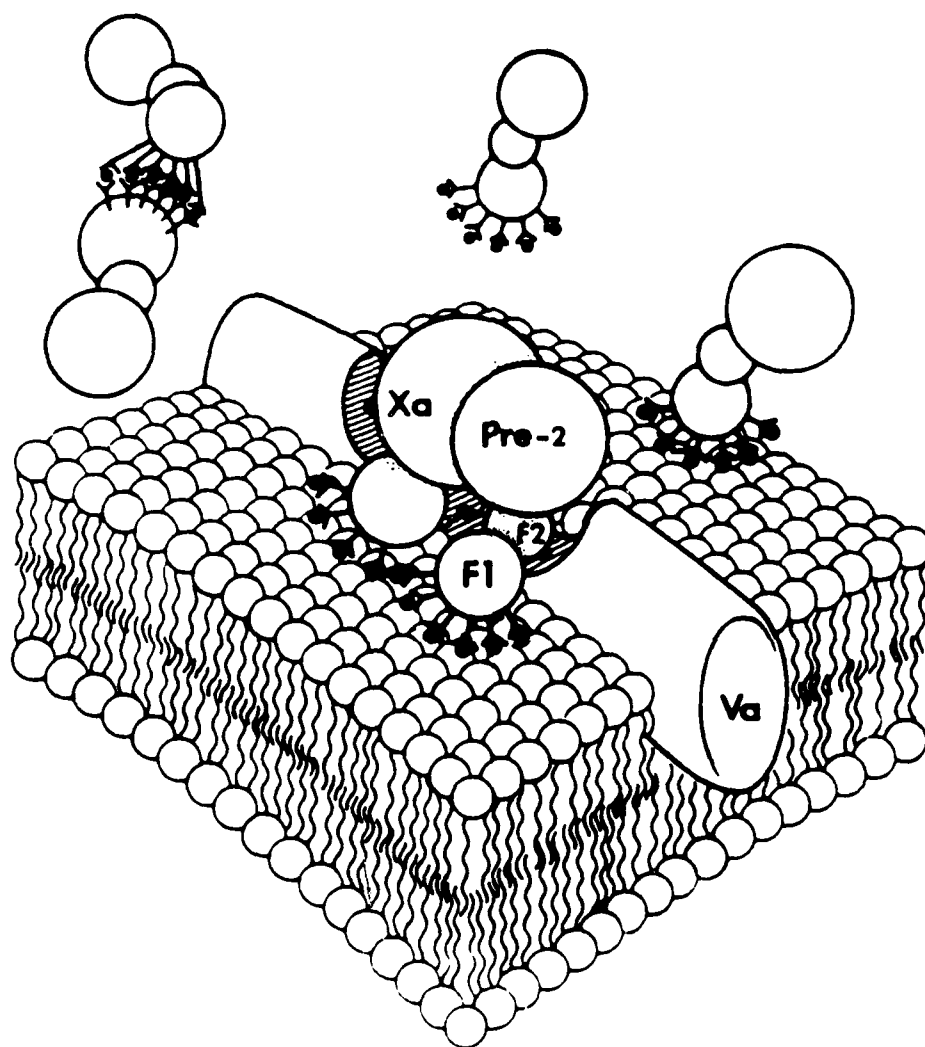


Figure 2

Figure 2. Model of the activation of prothombin by the prothrombinase complex. The large factor Va molecule is embedded in a phospholipid bilayer with factors Xa and II (prothrombin, shown with its three domains: Pre-2, F1, and F2) sequestered in close proximity to one another, within a cleft of the factor Va molecule. The dark dots represent calcium bridging, which may secure the enzyme and substrate within the cleft, thus providing optimum conditions for the rapid thrombin production necessary for hemostasis.

There is no apparent direct interaction between the prothrombinase substrate, prothrombin, and platelets. However, human and bovine factor Xa binds to a specific platelet receptor. This receptor appears to be absent in factor V-deficient human platelets, thus implying that factor V or Va may be the receptor (43,51). Exogenously added factor Va binds to isolated platelets with greater affinity than does the procofactor, factor V (43), in addition, factors V and Va are sequestered inside the platelet. Upon platelet activation, the sequestered factor V/Va may be released into the immediate microenvironment to significantly enhance the rate of thrombin production. Most likely the conversion of prothrombin to thrombin occurs on the platelet surface, which promotes fibrin formation, resulting in a clot at the site of vascular injury.

Tissue damage trauma activates the extrinsic coagulation pathway complex of factor VII, tissue factor, calcium and a phospholipid surface which, in turn activates factor X to Xa. Thus, there seems to be an interrelationship between the intrinsic and extrinsic activation pathways of the clotting cascade for activation of factor X to Xa. The frequently mentioned phospholipid surface can be provided by any of the cellular elements involved, or perhaps, by cellular debris. These complex formations result in tremendous increases in enzymatic rates in the clotting processes, and the accompanying decrease in rates of inactivation processes of the activated coagulation factors. Thus, complex formation accelerates processing of coagulation substrates, while it decreases the ability of coagulation inhibitors to stop enzymatic activations (11).

An interesting observation, demonstrating the role of endothelium in synthesizing plasma components with hemostatic activity, occurred when

endothelial cells were shown to synthesize portions of one of the essential cofactors, factor VIII: VIII:antigen (VIII:Ag) and VIII: von Willebrand factor (VIII:vWF)(12,13). For many years the site of synthesis of the multi-moiety VIII molecule had been speculative. In 1972 DeLos Santos and Hoyer (18), for the first time, described the presence of factor VIII:Ag in vascular endothelium using an immunofluorescence technique. A year later, other investigators (12,19,20) also demonstrated the presence of factor VIII:Ag in vascular endothelial cells, and indicated its possible role in hemostasis and thrombosis. In 1974, Jaffe et al. (21) discovered that endothelial cells release factor VIII:vWF into the culture medium. In addition, they later demonstrated the synthesis of factor VIII:Ag by RIA, and further demonstrated that the amount of factor VIII:Ag had increased in the culture medium in a linear fashion over a three day period (13). These studies provide information indicating that endothelial cells are capable of providing two of the important factor VIII complex moieties, VIII:Ag and VIII:vWF, though the site of synthesis of the coagulant portion of the molecule is still unknown. These observations have documented a direct role of endothelial cells in blood coagulation and hemostasis.

The other essential cofactor in the coagulation cascade is factor V, a high molecular weight (330,000 dalton, as determined by sedimentation equilibrium) single chain plasma protein, which, upon activation, serves as the cofactor for the optimum conversion of prothrombin to the blood clotting enzyme thrombin (11,15,16,22). Factor Va, cofactor for the reaction, is found in plasma in the form of a "pro"cofactor, factor V (17). The "pro"cofactor is converted to the active cofactor as a result of proteolytic cleavage by thrombin. Thrombin cleaves factor V in at least four places in the polypeptide chain, resulting in

several fragments (see Figure 3), two (94K and 74K) of which express cofactor activity in the prothrombinase complex. As indicated in Table 1, the deletion of factor Va from the prothrombinase complex results in a  $10^4$ -fold decrease in the rate of prothrombin conversion to thrombin, as compared to factor Xa alone. Thus, from a physiological standpoint, factor Va, as well as other prothrombinase components, must be present for a timely hemostatic response.

Factor V is an extremely labile protein, is found in human plasma at about 7 ug/ml, is very susceptible to protease action, and is, therefore, very difficult to isolate intact. It shares many physical and chemical properties with factor VIII. They both have high molecular weights, both are activated by thrombin, both serve as essential cofactors in enzymatic complexes, increasing the rate of enzymatic catalysis multifold when they are present, both have sedimentation coefficients in the 8-9S range, both bind to QAE cellulose and octyl-Sepharose, and both are inactivated by ethylenediaminetetraacetic acid (EDTA) (11).

The site of synthesis of factor V is unknown. Liver perfusion studies have indicated that many of the coagulation factors, including V, VIII, XI, XII and the vitamin K-dependent factors II, VII, IX and X, increase in the perfusate (23-27). However, the liver contains many cell types: parenchymal, biliary (epithelial), vascular (endothelial) and associated smooth muscle, and connective tissue. Immunofluorescence of liver sections has indicated that vitamin K-dependent factors are located in the liver parenchyma (23). Factor VIII:vWF and VIII:Ag have likewise been associated with vascular endothelium in the liver, as well as in other tissues (skin and kidney) (20,21).

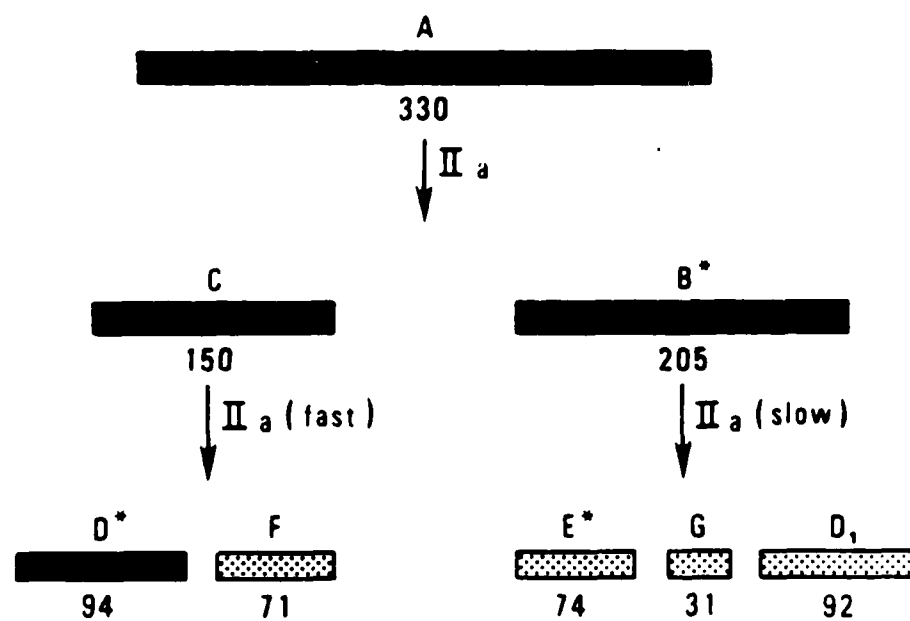


Figure 3

Figure 3. Activation of the Factor V Molecule. Activation is believed to occur through thrombin (IIa) cleavage of the 330,000 dalton single chain protein to yield two intermediates with apparent molecular weights of 205,000 dalton (B\*) and 150,000 dalton (C). The further cleavage of component C, also by thrombin, yields end products with molecular weights of 94,000 (D) and 71,000 (F). Most of the activity of Factor Va is temporally associated with cleavage of component C. Cleavage of the 205,000 dalton intermediate occurs at a slower rate yielding products with apparent molecular weights of 92,000 (D<sub>1</sub>), 74,000 (E\*) and 31,000 (G). Some increase in activity, *relatively minor by comparison* to cleavage of component C, may accompany the slower cleavages of component B.

The chemical and physical similarities between factors VIII and V were used as the basis for an hypothesis to determine if cultured bovine aortic endothelial cells synthesize coagulation factor V, since endothelium is known to synthesize coagulation factors VIII:Ag and VIII:vWF.



## EXPERIMENTAL PROCEDURE

### Materials and Methods

#### Cell Culture and Preparation of Primary Cultures:

Bovine aortas were obtained from Rock Dell Meats and Processing, Rock Dell, MN. Nalgene sterilizable containers were obtained from Fischer Scientific, Pittsburgh, PA. Monobasic and dibasic sodium phosphate, sodium chloride and glucose were from Fisher Scientific, Pittsburgh, PA. Powdered medium 199 was from M.A. Bioproducts, Walkersville, MD. Hyclone fetal bovine serum was supplied from Sterile Systems, Inc, Logan, UT. The following antibiotics were used: amphotericin (Fungizone), Squibb, Princeton, NJ; gentamycin (gar-mycin), Schering Corp., Kenilworth, NJ; penicillin, Parke-Davis, Santurce, PR; and streptomycin, Eli Lilly and Co., Indianapolis, IN. Type IV collagenase was obtained from Boehringer-Mannheim, Indianapolis, IN. Trypsin was from Sigma, St. Louis, MO. The medium 199 was solubilized in ultra-filtered water and pH adjusted, according to package instructions, immediately prior to filter sterilization through a Millipore Sterilizing 142mm filter unit, with pre-filter and 0.22 micron filter membrane, from a Gelman 11 liter pressure reservoir tank, using a 95% air-5% CO<sub>2</sub> gas mixture at 20 psi. The medium was then stored at 4°C, in sterile 500 ml medium bottles, for no longer than six months. Phosphate buffered saline was filter-sterilized and stored in the same manner. RPMI-1640 medium was prepared from the "Select-an-amine" kit from Gibco, Grand Island, NY, and was made deficient in methionine. All surgical equipment and supplies were obtained through Methodist Hospital, Rochester, MN. Culture dishes and tubes were obtained from CoStar, Cambridge, MA. Endothelial Cell Growth

Supplement (ECGS), for human endothelial cell cultures, was from Collaborative Research, Inc., Waltham, MA. Red Rim sterile filtration units were from Schleicher and Scheull, Inc., Keene, NH. Fresh frozen plasma for preparation of fibronectin, was purchased from Mayo Clinic Blood Bank, and fibronectin was prepared by the method of Roushlati et al (28). Cytodex III microcarriers were from Pharmacia Fine Chemicals, Piscataway, NJ.

Bovine aortas were obtained within thirty minutes of animal exanguination, and transported in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS), 1 mg/ml glucose, 10 ug/ml gentamycin, 1 ug/ml amphotericin at 4°C. Vessels were washed in the transport fluid to remove any blood components remaining. Accessory vessels were either sutured or tied closed, and one end of the aorta was sealed by stapling closed. A 37°C filter-sterilized solution of 0.6 mg/ml collagenase in PBS was placed into the vessel, and the remaining end clamped closed. The vessel was then placed into a 37°C PBS bath for a thirty minute incubation. The enzyme solution was decanted at the end of the incubation time, the vessel was filled with 37°C PBS, and mechanical agitation (gentle scraping with a rubber policeman or external mechanical blows from a large dull instrument or, the most successful method, manual manipulation by squeezing the PBS back-and-forth inside the sealed vessel) was used to dislodge the luminal endothelium. The cell suspension was decanted into sterile 50 ml Co-Star polypropylene conical centrifuge tubes, and centrifuged at 200 g for five minutes at room temperature. Supernatant PBS was then aspirated from the cell pellet, 37°C medium 199 supplemented with 10% FCS, 20 U/ml penicillin, 20 ug/ml streptomycin (hereafter referred to as medium 199 or medium) was added and the cell pellet suspended in the medium. The cell

suspension was then used to seed cells into 1) 100 mm polystyrene culture dishes, used for labelled amino acid incorporation studies, 2) 6-well cluster dishes, containing tissue culture washed and sterilized glass cover slips, which had been pre-coated with 30 ug/ml human fibronectin in medium 199, used for immunofluorescence studies, 3) 6-well cluster dishes , used to contain Cytodex 3 microcarrier beads for binding studies, and 4) 24-well cluster dishes, also used for binding studies. Culture supernatant, to be used for the bioassay and radioimmunoassay, was taken from all culture sources. Fresh medium was added the day after plating, and the cultures were allowed to become confluent (2-9 days), with medium changes every 3-4 days until confluency. A typical confluent culture is shown in Figure 4.

Human umbilical cords were obtained from Methodist Hospital, Rochester, MN, as soon as possible post-partum. Only sections which had not been damaged by clamps were used. At the delivery room suite, the umbilical vein was located and catheterized in both ends of the cord with sterile 3mm ball surgical steel catheters with leuer tips. One catheter was attached to a 20 cc syringe containing 37°C PBS-1 mg/ml glucose buffer, and the other had a 3 inch section of plastic tubing attached with clamp. The vessel was rinsed free of blood components using the syringe, and then maintained patent by slightly distending the vessel with pressure from the syringe, while the plastic tubing was clamped closed. Following this procedure, the cord was transported to the lab immersed in a 37°C PBS bath. The vessel was again rinsed with PBS-glucose, then filled to distention with 0.6 mg/ml collagenase-PBS-glucose. Collagenase was allowed to incubate 12-15 minutes inside the vessel, then drained into a conical tube by gravity. Syringes (20 cc) were attached to the

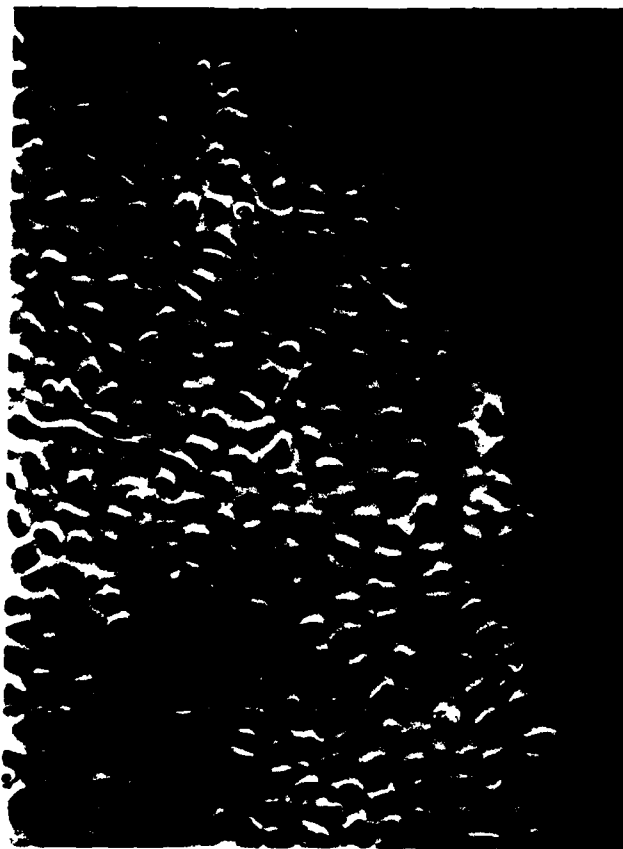


Figure 4

Figure 4. Confluent Bovine Aortic Endothelium. Endothelium was cultured from aortic tissue on tissue culture treated plastic surfaces. Cultured cells were allowed to reach confluence (cells covering entire surface of culture container), prior to use in any of the described experimental protocol, with medium changes (refeeding) every 3-4 days until confluence was attained. This primary culture exhibits the characteristic cobble-stone appearance peculiar to confluent endothelial cells in culture. (480x magnification, Zeiss Standard 18 microscope)

catheters, and 20 ml of PBS-glucose was forced back-and-forth through the vessel to shear sheets of cells loose. The buffer-cell suspension was drained into a sterile 15 ml conical centrifuge tube (Co-Star, Cambridge, MA), and centrifuged for 5 minutes at 1000 rpm. The cell pellet was suspended in medium 199 supplemented with 20% FBS, 20 U/ml penicillin, 20 ug/ml streptomycin, and 150 ug/ml ECGS, and seeded into Co-Star 6-well cluster culture dishes at 30-50% density of cell clumps per dish. Medium was changed the next day to remove debris and dead cells, and the culture was re-fed at 3-4 day intervals until confluent (7-10 days).

#### Isolation of Factor V from Bovine Plasma:

Bovine blood was obtained from animals exsanguinated at Rock Dell Meats and Processing, Rock Dell, MN. QAE-cellulose was from Schleicher and Schuell, Inc., Keene, NH. Sepharose CL-4B and octyl-Sepharose were from Pharmacia Fine Chemicals, Uppsala, Sweden and Cibacron blue dye was from Polysciences, Warrington, PA. Ultrapure ammonium sulfate was from Schwarz/Mann, Orangeburg, NY. Tris-base and soybean trypsin inhibitor (STI) were from Sigma, St Louis, MO. Beef lung heparin was from Upjohn, Kalamazoo, MI. Benzamidine-HCl was obtained from Aldrich, Milwaukee, WI. All other materials were analytical grade. QAE buffer was 0.025 M Tris-HCl, 5 mM  $\text{CaCl}_2$ , 1 mM benzamidine, pH 7.5.

The method of isolation of bovine factor V was as described by Nesheim et al. (33). No more than nine liters of blood were collected, by catching the blood flowing from an exsanguinated animal, into a plastic pail containing one liter of anti-coagulant. The anti-coagulant consisted of 2.85% trisodium

citrate, 10 mM benzamidine-HCl hydrate, 0.02% STI and 2.5 U/ml heparin. Collected blood was thoroughly mixed with anticoagulant, then placed into a ten liter carboy for immediate transport. Blood from two animals was collected for the isolation procedure to be described, and processing began as quickly as possible, with transportation time being the limiting factor. Plasma was separated from cells using a DeLaval cream separator, at a flow rate of one liter per minute at 4°C. Approximately ten liters of plasma was collected into a 15 liter plastic container. Then, 1 M BaCl<sub>2</sub> (80 ml/liter of plasma) was added via a separatory funnel over 20-30 minutes at 4°C with stirring, followed by 50% (w/v) polyethylene glycol (PEG) 6000, added in the same manner, to a final concentration of 4%. When all the PEG had been added, the mixture was stirred 30 minutes longer. This suspension was centrifuged at 6000 g for 20 minutes at 4°C using 0.5 and 1 liter centrifuge bottles in three standing centrifuges.

About 1000 ml QAE resin was washed in a 2 liter coarse scintered glass funnel with the following solutions: 1) 1 L 1 M NaCl, 2) 2 L distilled water, and 3) 2 L QAE buffer using moderate vacuum. The caramel-colored supernatant was decanted into a 20 gallon plastic container, and placed onto a heavy duty stirrer with a large (3 inch) stir bar. The equilibrated QAE resin was added to the slowly stirring supernatant, followed by cold dH<sub>2</sub>O and cold QAE buffer, in volumes equal to the supernatant volume (usually about 10 liters), and allowed to stir for about 45 minutes at 4°C. The QAE resin was then allowed to settle for one hour, supernatant fluid was siphoned off, the resin transferred into a 2 liter coarse scintered glass funnel, and placed onto a 4 liter Erlenmeyer vacuum flask, with partial vacuum, to pack the resin. After packing, a piece of

Whatman #1 filter paper was layered on top of the resin, followed by washing with 2 liters of 0.02 imidazole-HCl, 5 mM  $\text{CaCl}_2$ , pH 6.5, then 2 liters of the same buffer plus 0.1 M NaCl. Factor V was eluted with the same buffer plus 0.3 M NaCl. The eluate was collected in 3-4 fractions of about 500 ml each. Factor V eluted very sharply, and was usually found in one or two of these fractions. Washing and elution were accomplished at room temperature. Ultrapure ammonium sulfate (0.361 gm/ml) was added to the eluate and stirred for 30 minutes at 4°C. This suspension was centrifuged in two 250 ml plastic centrifuge bottles in a swinging bucket rotor (Sorvall HS-4) at 10,000 g for 20 minutes at 4°C. Supernatants were discarded, and more suspension added, until all the suspension had been centrifuged. The two pellets were stored overnight, upside down at 4°C. The QAE resin was washed with 0.02 M imidazole-HCl, 5 mM  $\text{CaCl}_2$ , 1 M NaCl, pH 7.5, and stored in 1 M NaCl at 4°C for future use.

An octyl-Sepharose column (4x32 cm) was prepared for use the next day by washing with 1) 5% Triton X-100, 2) 90% methanol (degassed), and 3)  $\text{dH}_2\text{O}$  (also degassed). All octyl-Sepharose chromatography procedures were accomplished at room temperature. The next day the octyl column was equilibrated with 0.01 M tris-borate, 1 M NaCl, 1 mM  $\text{CaCl}_2$ . The pelleted sample was dissolved in about 100 ml of equilibration buffer, and applied to the column. When all of the sample had been loaded onto the column, flow was stopped for about 30 minutes, then started, with a flow rate of about 7 ml/minute, collecting 10 ml fractions. Absorbance of fractions were read until  $A_{280}$  was less than 0.1. Elution was accomplished using a 0—0.6 M NaCl gradient in octyl buffer. Peak fractions were collected and pooled, then, ammonium sulfate (0.435 gm/ml) was added and stirred at 4°C for 30 minutes. The suspension was



pelleted by centrifugation at 10,000 g for 20 minutes at 4°C, and stored overnight (or over the weekend if necessary).

The pellet was dissolved in 100 ml 0.01 M tris-borate, 1 mM CaCl<sub>2</sub>, and applied to a Cibacron blue-Sepharose column (2.4x20 cm), which had been equilibrated in the same buffer. The column was washed with the same buffer until A<sub>280</sub> was less than 0.01. Elution was accomplished, using the same buffer with a 0—0.6 M NaCl gradient, collecting 10 ml fractions. Factor V peak fractions were pooled, chilled to 0°C, and precipitated with ammonium sulfate (0.435 gm/ml), while stirring for about 30 minutes at 4°C. The suspension was centrifuged in a swinging bucket rotor at 10,000 g for 20 minutes at 4°C. The pellet was carefully drained, then dissolved in 5-10 ml 0.01 M tris-borate, 1 mM CaCl<sub>2</sub>, 50% glycerol, and stored, in glass, at -20°C.

Factor V levels were determined throughout the isolation, using the bioassay as described below.

#### Factor V Bioassay:

Bioassays were done in 12 x 75 mm borosilicate (glass) clotting tubes from Fisher Scientific, Pittsburgh, PA. Samples to be bioassayed were diluted in 0.02 M imidazole, 0.15 M NaCl, pH 7.4, imidazole was from Eastman Kodak, Rochester, NY, and sodium chloride was from Fisher Scientific, Pittsburgh, PA. Factor V-deficient plasma was prepared by passing fresh human plasma over a human anti-factor V antibody-Sepharose resin, as per Katzmman et al. (29). Rabbit brain thromboplastin was prepared by the method of Bowie et al. (30), and kindly provided by same, and 0.025 M CaCl<sub>2</sub> was prepared from CaCl<sub>2</sub>, Fisher Scientific, Pittsburgh, PA. Bovine thrombin was prepared by the method

of Lundblad et al. (31) and was obtained from laboratory supplies. Triton X-100, for cell solubilization prior to bioassay, was from Packard Instrument Co.

A two stage bioassay, as described by Nesheim et al. (32), which detects the procofactor separate from the cofactor, was performed daily on 1:1 dilutions of cell supernatant. An 0.5 ml sample of cell supernatant was taken from cultures, and stored in a polystyrene covered 5 ml test tube. Two 25 ul aliquots were removed, and each placed into borosilicate clotting tubes containing 25 ul of 0.02 M imidazole-HCl, 0.15 M NaCl, pH7.4. The first stage assay was performed on one aliquot by adding 50 ul Factor V-deficient human plasma, 50 ul rabbit brain thromboplastin, and 50 ul 0.025 M  $\text{CaCl}_2$ , and rocking the tube back and forth in a 37°C water bath, while watching for a thrombin clot to form. The second stage was performed on the other aliquot, by adding 5 ul of a 10 U/ml solution of bovine thrombin, incubating for 60 seconds in the 37°C water bath, then immediately performing the stage one assay procedure described above. In addition to the supernatant, cells were solubilized using 1% triton, and used as a substrate in the assay. 1% triton had been determined by Tracy et al. (42,43) not to interfere with the assay. The assay was also performed on unused FBS-supplemented medium, to provide a background control. For antibody inhibition studies, polyclonal burro anti-bovine factor V and three purified monoclonal antibodies to factor V were used. Two of the monoclonals, IIC3 and IVC6, are inhibitory antibodies, and IB6 is a noninhibitory antibody. For the polyclonal studies, nonimmune burro serum was used as a control.

### Preparation of $^{125}\text{I}$ -Factor V:

Bovine factor V was prepared by the method of Nesheim et al. (32,33), and obtained from laboratory stock. TAGIT (N-succinimidyl-3-(4-hydroxyphenyl)-propionate) was from Sigma Chemical Co., St. Louis, MO. Chloramine T and sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) were obtained from Mallinckrodt, St Louis, MO. Dimethyl formamide was from J.T. Baker Chemical Co., Phillipsburg, NJ.  $\text{Na}^{125}\text{I}$  (13-17 mCi/ug) was obtained from Amersham. Cibacron-blue chromatography resin was prepared from Pharmacia CL-4B Sepharose by the method of Travis et al. (41), and was obtained from laboratory supplies. All other reagents used for labelling were analytical grade.

One milligram of bovine Factor V, prepared by the method of Nesheim et.al.(33), in glycerol-water, was dialyzed versus 0.1 M sodium borate, pH 8.5 for four hours with one change of dialysate. After dialysis, spectrophotometric determination of protein concentration was made using an extinction coefficient of 0.96 for bovine Factor V. The protein was kept at 4°C awaiting Bolton-Hunter labelling procedure (44).

The following reagents were prepared immediately prior to use: TAGIT- one milligram dissolved in 1 ml benzene, then diluted to 10 ug/ml in benzene, and kept at 4°C until used; chloramine T- 5 mg/ml in 0.2 M sodium phosphate buffer, pH 7.5; and sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ )-12 mg/ml in 0.2 M sodium phosphate buffer, pH 7.5. Acid washed glass conical centrifuge tubes were used for the reaction, with reagents carefully placed into the tip of the tube in the following order, and each addition followed by vigorous vortexing: 1) 400 ng TAGIT in benzene with benzene evaporated via vacuum pump; 2) chloramine T - 10 ul; 3) 5 mCi  $\text{Na}^{125}\text{I}$  (13-17 mCi/ug) brought up to 50 ul in 0.2 M sodium

phosphate buffer, pH 7.5; 4)  $\text{Na}_2\text{S}_2\text{O}_5$  - 10  $\mu\text{l}$ ; 5) potassium iodide (20 mg/ml in sodium phosphate buffer) - 10  $\mu\text{l}$ ; 6) dimethyl formamide-10  $\mu\text{l}$ ; and 7) 0.5 ml benzene to extract. The complete reaction mixture was vigorously vortexed, and the top benzene layer was carefully removed and placed into a clean glass tube. Radioactivity was counted to determine labelling efficiency. Sufficient Bolton-Hunter reagent was placed into another clean glass tube to label the protein at 2 mCi/mg, and the benzene evaporated by vacuum pump, leaving the Bolton-Hunter reagent adherent inside the tube. The Factor V was immediately added, gently vortexed, and kept at  $4^\circ\text{C}$  for 20 minutes.

The reaction mixture was quenched with 50  $\mu\text{l}$ /ml reaction volume with 2 M glycine, 0.1 M sodium borate buffer. The quenched reaction mixture was diluted 1:5 with 0.01 M tris-borate- $\text{CaCl}_2$ , pH 8.3 buffer, and applied to Cibacron-blue resin, prepared by the method of Travis et al. (41). The flow-through was collected into one tube, and absorbance read to determine the quantity of protein adherent to the resin. The Cibacron resin was then washed with 0.01 M tris borate- $\text{CaCl}_2$  buffer, until the absorbance<sub>280</sub> was zero, followed by 0.1 M NaCl in the tris borate- $\text{CaCl}_2$  buffer, until the absorbance<sub>280</sub> was zero. The resin was eluted with 0.5 M NaCl in tris borate- $\text{CaCl}_2$  buffer, and 1 ml fractions were collected. The absorbance<sub>280</sub>, and gamma counts, of each fraction were determined, peak tubes dialyzed vs tris-glycerol, and the dialyzed bovine  $^{125}\text{I}$ -Factor V was stored in a glass vial at  $-20^\circ\text{C}$ . A representative chromatogram is shown in Figure 5.

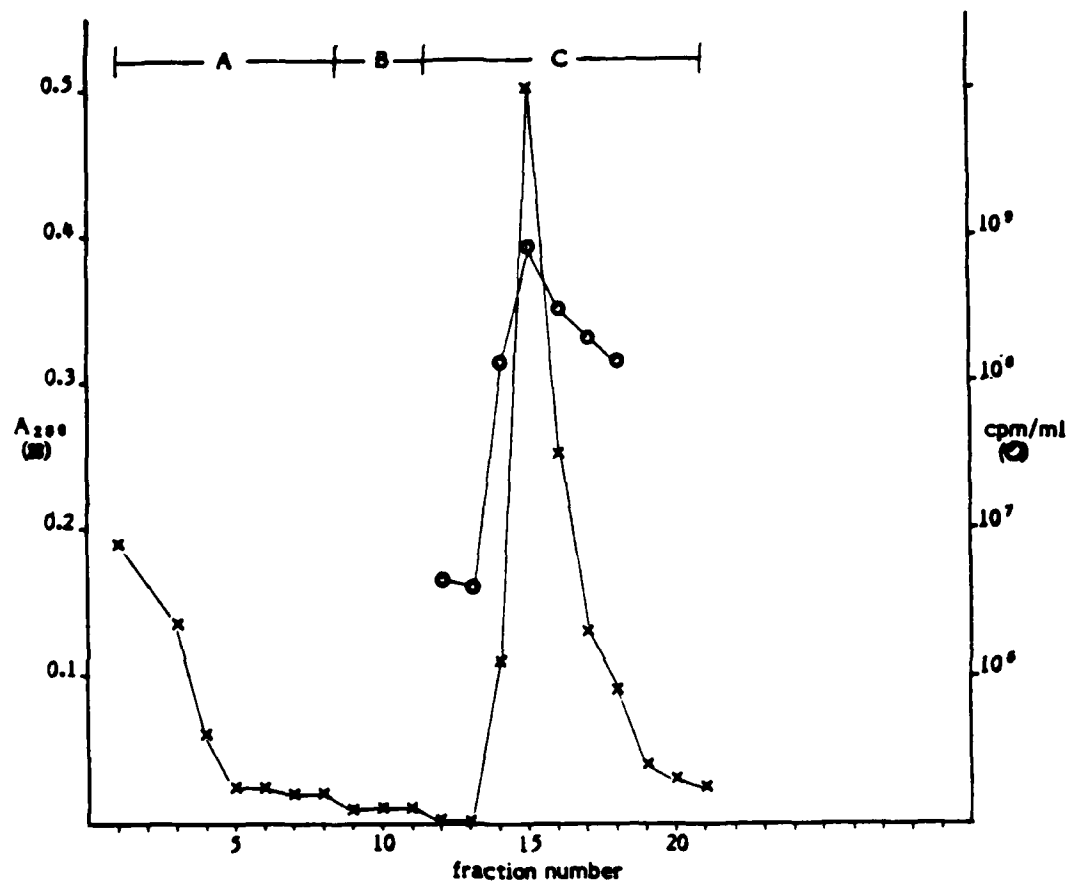


Figure 5

Figure 5. Iodination of Factor V. The Bolton-Hunter procedure was used to radiolabel Factor V with  $^{125}\text{I}$ . Shown is a representative chromatograph from separation of labelled protein and free Bolton-Hunter reagent on a guanidine hydrochloride washed Cibacron blue-Sepharose resin. Buffer A, 0.01 M tris borate- $\text{CaCl}_2$ , washed the applied labelled protein solution until the  $A_{280}$  was "0", which was followed by buffer B, 0.01 M tris borate- $\text{CaCl}_2$ , 0.1 M NaCl, until the  $A_{280}$  was "0". The resin was then washed with buffer C, 0.01 M tris borate- $\text{CaCl}_2$ , 0.5 M NaCl, to dissociate the protein from the resin and 1 mL fractions were collected. Absorbance $_{280}$  and gamma irradiation was determined for each fraction and peak fractions with highest specific activity were retained for use in experimental protocols.

#### Factor V Radioimmunoassay:

Burro anti-bovine Factor V-IgG was obtained from laboratory stocks of an ammonium sulfate precipitate of immune burro sera. Normal burro sera was obtained from the Institute Hills Farm, Mayo Clinic/Foundation, Rochester, MN. Assay buffer was 0.075 M tris-HCl, 0.075 M NaCl, 1% BSA (RIA grade from SIGMA), pH 7.0. All other reagents were analytical grade.

The burro anti-bovine factor V IgG was titrated, to determine that dilution which precipitated 30-40% of the antigen (factor V), see antibody titration curve Figure 6. However, when a standard curve was prepared using 5 ng of  $^{125}\text{I}$ -factor V, a dilution of 1:3000 provided a more useable curve than had the 30% point of the titration curve, see Figure 7. The high specificity of this antibody for factor V antigen made the RIA a definitive means of bovine factor V detection, as had been previously shown by Tracy et al. (43). The amount of  $^{125}\text{I}$ -factor V used was determined by comparing the slopes of standard curves, using 5, 10 and 20 ng of  $^{125}\text{I}$ -factor V. In the straight region of the binding curves, the slope for 5 ng provided definitive determination of antigen levels, as well as providing determination over a wider range of levels, see Figure 8.

Radioimmunoassay of samples, which had been taken from the culture supernatants, was accomplished using the double antibody technique described by Tracy et al. (42,43). Into plastic tubes (1.0 x 6.0 cm) were added 0.2 ml cell supernatant, 0.2 ml  $^{125}\text{I}$ -bovine Factor V (5 ng) in RIA buffer (0.075M tris, 0.075 M NaCl, 1% BSA, pH 7.0), and 0.2 ml of a 1:3000 dilution of burro anti-bovine Factor V IgG (dilution used precipitated 30% of antigen when antibody was titrated) diluted in 1:40 normal burro serum. The 1:40 normal burro serum was added, to provide sufficient IgG to form a manageable precipitate, after

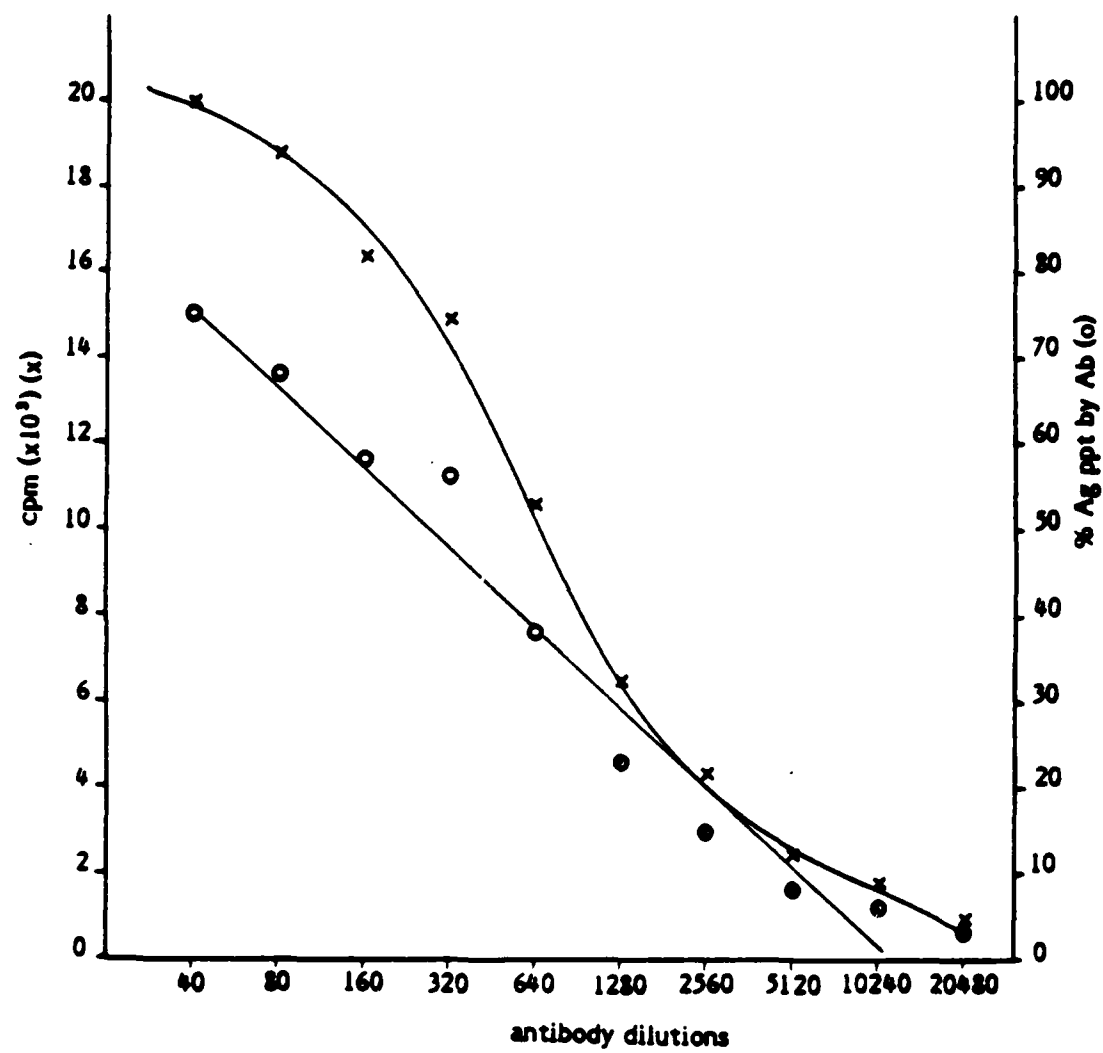


Figure 6



Figure 6. Titration of Burro Anti-bovine Factor V IgG. The ammonium sulfate precipitated polyclonal burro IgG, which had resulted from immunization of a burro with purified bovine factor V, was titrated, using a serial dilution method. The buffer and method used was the same as the radioimmunoassay.  $o$  = percent antigen precipitated by the polyclonal antibody (IgG);  $x$  = cpm ( $\times 10^3$ ) of  $^{125}\text{I}$ -factor V in the precipitate.

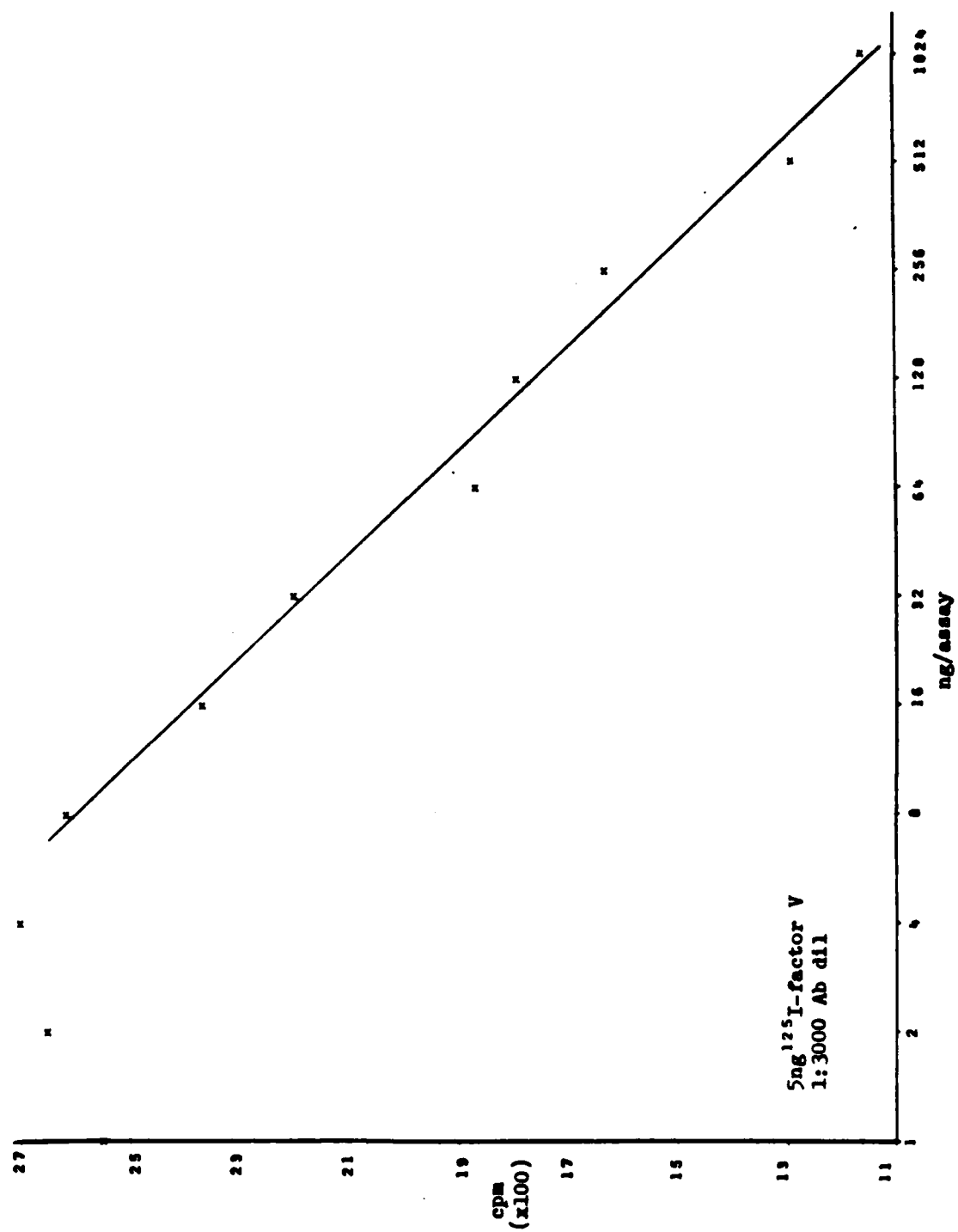


Figure 7

Figure 7. Standard Curve for Radioimmunoassay. This standard curve is representative of standard curves run simultaneously with experimental samples for the radioimmunoassay protocol. As indicated, this standard curve was accomplished using 5ng of  $^{125}\text{I}$ -factor V and a 1:3000 antibody dilution. This dilution provided a linear curve for samples containing low concentrations of factor V.

the addition of the goat anti-burro IgG. The tube was vortexed, and allowed to incubate for thirty minutes at 37°C. Goat anti-burro IgG (0.2 ml) was then added, with sufficient titer to precipitate all the burro IgG present. The tubes were incubated over night at 4°C, and then centrifuged, at room temperature, for five minutes at 400 g. The precipitated pellet was washed twice with RIA buffer, and centrifuged, forming a pellet. The pellet was counted in a Beckman 8000 gamma counter. A standard curve was accomplished, using known amounts of purified Factor V in concentrations from 1-1024 ng/assay tube, with a zero control point. Data was plotted on semi-log graph paper, cpm/assay vs. ng/assay, and experimental data interpolated from the standard curve.

#### Immunofluorescence:

The buffered acetone-formalin fixer was prepared by the method of Yam et al. (34). Wash buffer was 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4, prepared using monobasic and dibasic sodium phosphate to adjust the pH appropriately. RIA grade bovine serum albumin (BSA), from SIGMA, was used to make the PBS wash buffer 1% BSA, for diluting antibodies and as the first dip-wash. Behring polyclonal rabbit anti-human factor VIII:Ag IgG was from Cal-Biochem, Sommerville, NJ. Non-immune rabbit IgG, purified from rabbit plasma by the method of Ey et al. (35), burro anti-bovine Factor V/Va ( ) and burro nonimmune serum were obtained from laboratory stock. Mouse monoclonal antibodies, to bovine factor V, were prepared from ascitic fluid as described by Foster et al. (36) and kindly provided by Dr. W.B. Foster (Mayo Clinic, Rochester, MN). Nonimmune mouse IgG was prepared from mouse serum by ammonium sulfate precipitation, and generously donated by Dr. N.

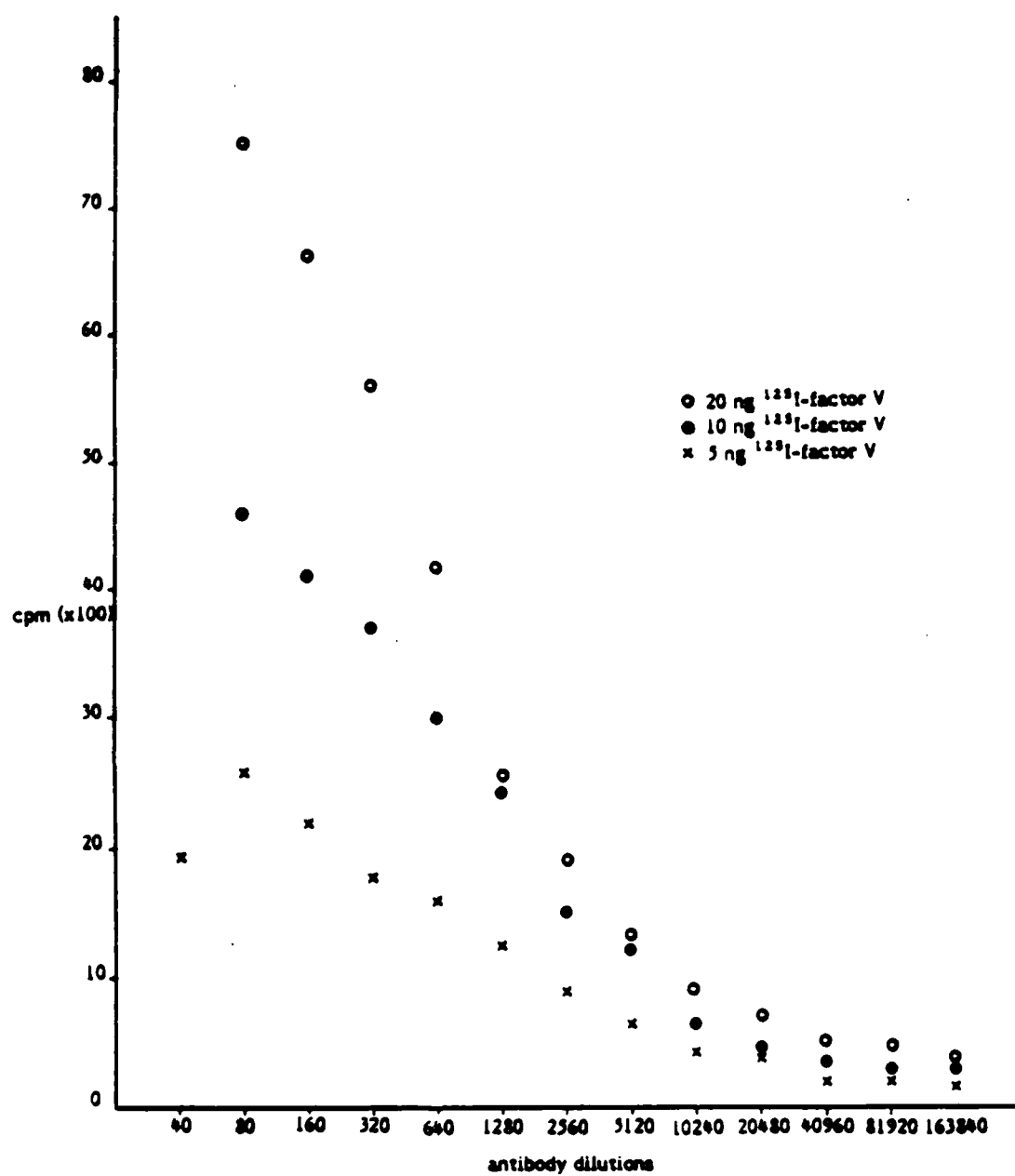


Figure 8

Figure 8. Standard Curves with Varying  $^{125}\text{I}$ -Factor V Concentrations. Varying concentrations of the radiolabelled factor V were examined in the radio-immunoassay to determine which concentration would provide the most linear standard curve over the widest dilution range. The binding curve for 5 ng proved to be the best of the three concentrations of factor V tested.

Gonchoroff (Mayo Clinic, Rochester, MN). Anti-mouse, anti-rabbit, and anti-horse fluorescein isothiocyanate-conjugated antibodies were obtained from Cappel Laboratories, Cochranville, PA. Kodak Ektachrome 400 film was from Eastman Kodak, Rochester, NY. All other reagents were analytical grade.

An indirect immunofluorescence technique was used, which involved the use of a primary antibody directed against the protein in question, and a secondary fluorescein isothiocyanate (FITC) conjugated antibody directed against the primary antibody (45-46). In preparation for the immunofluorescence, BAE were cultured on fibronectin-coated glass cover slips. Glass cover slips had been prepared for tissue culture by placing into a coverslip rack and soaking in ethanol-acetone (1:1) for one hour, rinsing for one hour under running de-ionized water, drying in a 100°C oven one hour, and sterilizing by steam autoclave. Cover slips were then placed into the wells of a 6-well (35 mm diameter wells) Co-Star cluster dish. One milliliter of a 30 ug/ml solution of human fibronectin, in unsupplemented medium 199, was placed onto the cover slips and incubated, for one hour, at room temperature, then aspirated off and discarded. Cells, which had been harvested from an aorta and suspended in 10% FBS supplemented medium 199, were seeded onto the precoated cover slips at high density (50% of culture surface covered by cell clumps). When cells attained confluency, cover slips were dip-washed in three room temperature PBS washes, carefully drained, fixed in 4°C buffered acetone-formalin for 90 seconds, and dip-washed in three 4°C PBS washes. Cells, fixed on the cover slips, were either used immediately, or stored in cover slip coplin jars at -70°C, for up to 10 days. If cells had been stored frozen, temperature conditioning, one hour at -20°C, followed by 30 minutes at 4°C in the sealed coplin jar, was

accomplished prior to rehydration. Cells were rehydrated in a sealed moist chamber for one hour at 37°C. From this point on, cells were continuously maintained in a moist environment. Following rehydration, cover slips were washed in room temperature PBS for three-five minute washes, then dip washed in PBS-1% BSA, followed by two PBS washes. Cover slips were carefully drained of excess PBS, and placed onto glass rods, cell side up, inside a moist chamber, and 200 ul of the primary antibody dilution was carefully pipetted onto the surface of the cover slip. Primary antibodies were diluted 1:50, from 8-10mg/ml IgG stock solutions, into PBS-1% BSA from the following stock immunoglobulins: 1) rabbit anti-human Factor VIII-VWF, 2) non-immune rabbit IgG, 3) mouse monoclonal anti-bovine Factor V/Va IgG (primarily IB6 and IIIA5, though other mouse monoclonal antibodies were tried unsuccessfully), and 4) non-immune mouse IgG. Cells were incubated with primary antibodies for thirty minutes. Then excess primary antibody was removed by washing the cover slips, in coplin jars, with three PBS washes. The cover slips were again dip-washed in PBS-1% BSA, and two PBS washes, carefully drained of excess PBS, and replaced onto the glass rods in the moist chamber. Secondary fluoresceinated (FITC) antibodies, anti-mouse and anti-rabbit, were diluted 1:200 in PBS-1% BSA, 200 ul was carefully applied to the cells, and incubated for thirty minutes at room temperature. The excess FITC-antibody was removed by washing in three five minute PBS washes, the moist cover slip was wet-mounted on a microscope slide with glycerol-PBS (1:2), and sealed with clear nail polish. Slides were stored overnight, at 4°C, covered with aluminum foil, before microscopic examination. Slides were brought to room temperature for one hour, then viewed, and photographed, with a Zeiss Standard 18



microscope, equipped with appropriate excitation and barrier filters for FITC, using a 40x oil immersion lens. Photographs were taken of positive, and negative, samples with Kodak Ektachrome 400 film, using a uniform exposure time of 60 seconds.

The rationale for a uniform exposure time was based on providing optimum film exposure to the fluorescent signal, while minimizing photo-bleaching of FITC, and the sixty second exposure time optimally met both requirements. Behring anti-factor VIII-vWF antibody was chosen as a positive control, because vWF-fluorescence is an endothelial characteristic used for identification. Rabbit and mouse non-immune IgG were chosen as negative controls, to ensure that any non-specific reaction to immunoglobulins could be accounted for, and compared, as background, to positive fluorescence.

Problems encountered with the immunofluorescence technique included cell culture variability in adhering to the glass coverslips, and monoclonal antibodies being specific for a particular domain of the factor V molecule, which, dependent upon how endothelial cells present factor V/Va, may not have been available for antibody interaction.

Immunofluorescence experiments were also carried out on bovine aortic endothelium (2<sup>0</sup> passage) grown in Falcon 35 mm tissue culture dishes (Oxnard, CA). Three days post-confluence, the cells, in the dishes, were rinsed three times at five minute intervals with successive 1 ml volumes of wash buffer (PBS), and fixed for 10 minutes at 4<sup>0</sup>C with cold 70% ethanol. The ethanol was removed, and the cells were washed, as mentioned above. Approximately 0.5 ml burro anti-bovine Factor V/Va IgG was diluted 1:250 in PBS-1% BSA, containing 1:250 nonimmune burro serum, and applied to the fixed cells for 30

minutes in a moist chamber at ambient temperature. As a control, nonimmune burro serum, diluted 1:250, was similarly applied. The cells were washed, and normal goat serum, diluted 1:20 in PBS-1% BSA, was applied for 15 minutes. Another PBS wash was followed by application of fluorescein conjugated goat anti-horse antiserum. After incubation and washing, coverslips were affixed over several drops of glycerol:PBS (1:1). The cells were inspected, and photographed, via epi-illumination fluorescence microscopy.

#### Description of Murine Monoclonal Antibodies

Murine monoclonal antibodies, for immunofluorescence and immunochromatography isolation, were prepared by Foster et al. (36), and generously provided by same. The following is a brief description of the monoclonal hybridoma techniques used to obtain anti-bovine factor V/Va antibodies.

BALB/c mice were initially immunized with 100 ug factor V/Va and inactivated factor Va in complete Freund's adjuvant, followed by two 100 ug injections in 0.02 M imidazole-HCl, 0.15 M NaCl, pH 7.4, at 21 day intervals. Spleen cells from these mice were then fused with murine NS-1 myeloma cells (29,65), and plated on 24 well cluster dishes containing mouse peritoneal-wash feeder cells. After 10-14 days, culture fluids, from wells containing cell growth, were assayed for antibodies to factor V/Va using a solid phase RIA. In this solid phase RIA, murine antibodies to factor V/Va were detected by the addition of culture fluid samples to polystyrene tubes, which had been coated with rabbit anti-mouse immunoglobulin. After a six hour incubation, specific murine antibodies were detected by the addition of iodinated factors V and Va. Antigen was incubated with the stationary antibodies for six hours, then

unbound antigen was removed by washing with buffer. Bound radioactivity determined efficacy and specificity of antibodies. The most reactive wells were subcultured (subcloned), and samples frozen for future growth as ascites tumors for the preparation of high titer antibodies. Wells, chosen for subcloning, were selected on the basis of their reactivity with iodinated factors V and Va, i.e., reactivity to factor V > Va, factor V = Va, or factor Va > V.

Monoclonal antibodies from all three reactivity groups were chosen for immunofluorescence. And a monoclonal antibody with equal reactivity to both factors V and Va was immobilized on CNBr-activated Sepharose CL-4B, as per March et al. (47), for immunochromatography isolation experiments.

#### In Vitro Clotting Studies:

Human fibrinogen was obtained from either IMCO Corp., Ltd. or Kabi Diagnostika, Stockholm, Sweden. Phosphatidyl choline phosphatidyl serine vesicles were prepared by the modified method of Barenholz et al. (37), as described by Bloom et al. (38), and generously supplied by Drs. D. Higgins and/or M. Tucker, Mayo Clinic/Foundation, Rochester, MN. Factor Xa was prepared by the modified method of Downing et al. (39), and generously supplied by Dr. P.B. Tracy, Mayo Clinic/Foundation, Rochester, MN. Bovine factor V was prepared by the method of Nesheim et al. (32,33), and obtained from laboratory supplies of factor V. Bovine prothrombin was prepared as described by Bajaj and Mann (40), and was obtained from laboratory stock prothrombin. All other reagents were analytical grade.

Aortas were obtained as previously mentioned, and stored in 4°C transport buffer, rinsed free of blood elements with 4°C transport buffer, and split

longitudinally to expose the luminal surface of the vessel. The vessel was then placed into room temperature reaction buffer, 5 mM HEPES, 0.15 M NaCl, pH 7.4. Reagents for clot formation were used to provide the following concentrations in a 100  $\mu$ l reaction mixture 1) 0.3 mg fibrinogen (IMCO or Kabi), 2) 3 mM  $\text{CaCl}_2$ , 3)  $40 \times 10^{-6}$  M PCPS vesicles, 4) 0.01 mg thrombin, 5)  $1 \times 10^{-8}$  M Factor Xa, and 6)  $1 \times 10^{-8}$  M and  $1 \times 10^{-9}$  M Factor Va. Immediately prior to use, Factor V was activated to Factor Va, by adding 10  $\mu$ l of 200 U/ml thrombin to the Factor V stock reagent and incubating for five minutes at  $37^\circ\text{C}$ . Reagents were added sequentially to reaction areas on 1) plastic 24-well cluster dishes, 2) glass microscope slides, and 3) aortic lumen. Sites were chosen on the aortic lumen which were at least 1 cm from any cut edge, not involving an accessory vessel aperture, and which could accomodate a 100  $\mu$ l reaction mixture within a discrete area of approximately 2 cm diameter.

Control reactions, 100  $\mu$ l each, were carried out in plastic polystyrene negatively charged tissue culture dishes, as well as on glass microscope slides. Intrinsic clotting of the fibrinogen was determined by the reaction of fibrinogen,  $\text{CaCl}_2$  and buffer on the plastic, glass, and aortic surfaces. The other reagents, required for optimum clot formation, were added in the following order to sequential reaction mixtures: 1) prothrombin, 2) Factor Xa, and 3) Factor Va. In addition, one reaction was accomplished, using PCPS vesicles to provide a positive control on plastic and glass.

The same series of additions were made on the aorta explant luminal surface, less the PCPS. A plastic pipette tip was used to provide movement and mixing of the reagents, as well as to provide a method for visualization of fibrin strand, or clot, formation. Careful movement of the pipette tip, parallel to the

luminal surface, through the reaction mixture, ending with an upward motion, was used to determine initiation of clotting, while trying not to damage the endothelium.

#### Factor Va Binding to Cultured BAE Cells:

Dextran conjugated microcarriers (Cytodex 3) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Tyrodes buffer was prepared as follows: 0.05 parts stock solution I (160 mg/ml NaCl, 4 mg/ml KCl, 20 mg/ml NaHCO<sub>3</sub>, 1.16 mg/ml NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O), 0.01 parts stock solution II (100 mM MgCl<sub>2</sub>), 0.01 parts stock solution III (100mM CaCl<sub>2</sub>), 0.75 parts distilled H<sub>2</sub>O, 0.7 g Pentex BSA (defatted), 0.2 g dextrose, 5 mM HEPES and brought up to 1.0 parts with distilled H<sub>2</sub>O. <sup>125</sup>I-bovine factor V was used at 50 ng/ml in the reaction mixture. DAPA was used at 30 uM to inhibit thrombin proteolytic activity. Time point samples were spun through Apiezon oil (E.J. Biddle & Co.):butylphthalate (1:9 ratio). Samples were spun through the oil, in Ependorf microfuge tubes, in a Brinkman-Ependorf centrifuge, at 18,000 rpm. Samples of reaction cells were dissociated from the cytodex 3 microcarriers with 0.5 ml of 2.5 mg/ml trypsin, 20 ug/ml EDTA in 5 mM HEPES buffer, and cells were counted in a hemacytometer.

To study the binding properties of endothelium for Factor Va, BAE cells were grown on Cytodex 3 microcarriers (100-micron diameter dextran spheres coated with collagen) in 6-well Co-Star cluster dishes, under previously mentioned medium conditions, until confluent. Control beads were also placed into the 6-well cluster dishes in the same medium, and under the same incubation conditions. Confluent and control microcarriers were removed from culture

conditions, and gently washed 2 times with Tyrodes-albumin-5 mM HEPES (buffer). Buffer was added to provide sufficient volume for eight 0.5 ml aliquots to be sampled for the binding procedure.  $^{125}\text{I}$ -bovine Factor V was activated by adding 1.5 U thrombin, and incubating for 5 minutes at  $37^{\circ}\text{C}$ ; then 50 ng/ml  $^{125}\text{I}$ -Factor Va was used in the reaction mixture for the binding studies. The reaction mixture was made 30  $\mu\text{M}$  DAPA to inhibit further proteolytic action by thrombin. The reaction was accomplished using both cells and control beads. Aliquots of 0.5 ml were taken at time points 1, 5, 10, 15, 20, 30, and 60 minutes after the  $^{125}\text{I}$ -Factor Va was added to the buffered reaction mixture. The aliquots were carefully layered onto 0.5 ml of Apiezon oil:butylphthalate (1:9) in Ependorf plastic conical centrifuge tubes, and centrifuged in an Ependorf centrifuge for 10 minutes at 18,000 rpm. Following centrifugation, the oil was removed from the bead pellet, and placed into marked polystyrene tubes. The section of the centrifuge tube containing the bead pellet was cut off with a razor blade, and the pellet was placed into a marked polystyrene tube. Both the supernatant oil, containing unbound  $^{125}\text{I}$ -Factor Va, and the cell-bead pellets were counted in a Beckman 8000 gamma counter. The remaining 0.5 ml of cells-beads, in buffer with the  $^{125}\text{I}$ -Factor Va, was counted as a control, for total counts, followed by digestion of the cells from the beads with trypsin-EDTA, and cell counting to determine the number of cells in the 0.5 ml reaction mixture.

#### DAPA Fluorescence Assay:

DAPA (dansylarginine N-(3-ethyl-1,5-pentanediy)amide) was prepared by the method of Nesheim et al. (64) and obtained from laboratory supplies. The

buffer used was 10mM HEPES, 11 mM glucose, 0.15 M NaCl, pH 7.35. Thrombin (IIa), factor Va, and factor Xa were obtained from laboratory supplies.

To determine if endothelial cells provide a surface for the prothrombinase complex to assemble upon, endothelial cells were grown to confluence on Cytodex 3 microcarriers (as mentioned above) and in plastic disposable spectrophotometry cuvettes (which had been gas sterilized and fibronectin coated). Cells were then used in a DAPA fluorescence assay. The buffer used for the assay was 10 mM HEPES, 11 mM glucose, 0.15 M NaCl, pH 7.4. Other reagents included 1 M  $\text{CaCl}_2$  used at 3 mM in reaction; bovine prothrombin (II) used at 0.1 mg/ml in the reaction; bovine Xa was titrated at concentrations from  $5 \times 10^{-9}$  to  $7.5 \times 10^{-8}$  M; bovine Factor Va (activated by thrombin) used at  $5 \times 10^{-9}$  M; DAPA (dansyl arginine piperidine amide) used at 4.5  $\mu\text{M}$  in reaction mixture; and PCPS vesicles for a control standard curve were used at 40  $\mu\text{M}$ . Prothrombin,  $\text{CaCl}_2$ , and DAPA were combined, and allowed to set at room temperature for 30 minutes prior to starting assays. To 1 ml of the prothrombin-DAPA- $\text{CaCl}_2$  solution, in the cuvette, was added 1) 0.5 ml of buffer (to the cells grown in cuvettes), 2) 0.5 ml of the Cytodex 3-endothelial cell suspension, or 3) 0.5 ml of buffer containing PCPS vesicles, to be used for standardization, and to obtain a maximal conversion rate. The following order of reagents were then added to the cuvette reaction mixture in the fluorometer, one at a time, followed by gentle mixing: bovine Factor Xa concentration titrated from  $5 \times 10^{-9}$  M to  $7.5 \times 10^{-8}$  M and bovine Factor Va (thrombin activated immediately prior to use) used at  $5 \times 10^{-9}$  M in the reaction cuvette. The change in fluorescence was determined using a Perkin-Elmer MPS-44A fluorometer.

#### S-2238 Chromogenic Assay:

H-D-Phe-Pip-Arg-NH-  $\text{-NO}_2 \cdot 2\text{HCl}$  (S-2238) was obtained from Kabi Diagnostika, Stockholm, Sweden. The same buffer as above was used. Prothrombin was obtained from laboratory supplies. "Prothrombinase" solution, for the reaction, was prepared using 0.15 mg/ml prothrombin and 4.5 mM  $\text{CaCl}_2$  in reaction buffer. Factors Va and Xa were obtained from laboratory supplies. Cells, for the reaction, had been cultured on Cytodex 3 microcarriers.

To further determine if cultured endothelial cells provided a phospholipid surface for prothrombinase complex attachment, a chromogenic assay for the detection of thrombin production (thrombin cleaves after arginine, thus releasing p-nitroaniline from the artificial thrombin substrate H-D-Phe-Pip-Arg-NH-  $\text{-NO}_2 \cdot 2\text{HCl}$  (S-2238)) was employed. Cells which had been grown on Cytodex 3 microcarriers, and cells which had been grown in spectrophotometer cuvettes, were used. The buffer for the S-2238 assay was 10 mM HEPES, 11 mM glucose, 0.15 M NaCl, pH 7.4. Cells were rinsed free of culture medium with buffer, then buffer was added to the cells to provide adequate volume for the assay. Other reagents for the assay included: bovine prothrombin (Factor II) at 0.1 mg/ml in the reaction; 1 M  $\text{CaCl}_2$  added to reaction to make it 4.5 mM  $\text{CaCl}_2$ ; bovine Factor V stock activated to Factor Va (with bovine thrombin) used to make reaction mixture  $5 \times 10^{-9}$  M Factor Va; bovine Factor Xa stock used to make reaction mixture  $5 \times 10^{-9}$  M Factor Xa; PCPS vesicles used from stock to make the standard curve reaction mixture 40  $\mu\text{M}$  PCPS. Reagents were added to endothelial cells grown on C-3 microcarriers, C-3 microcarriers with no cells, and to a PCPS control in the following order: buffer, prothrombin,  $\text{CaCl}_2$ , Factor Xa, and finally Factor Va. The reaction was allowed to proceed,



and 100  $\mu$ l samples were taken, at 1,2,5,10,15,30,60, and 90 minutes from the time Factor Va was added to the reaction, and placed into a test tube containing 1  $\mu$ l of 0.5 M  $\text{Na}_2\text{EDTA}$  (to stop thrombin's proteolytic activity). From a freshly prepared 1 mM S-2238 stock, a 1:20 dilution was made, in tris-saline, for an assay concentration of 0.05 mM S-2238. For each assay, 0.6 ml of S-2238 dilution was used with 10  $\mu$ L of the EDTA-reaction mixture in an 0.5 mm quartz cuvette, and the absorbance read at 405 nm in a spectrophotometer. An aliquot of the beads were taken, and trypsin-EDTA digestion of the cells was done to permit cell counting in a hemacytometer. In addition, in the trial experimental protocol, stimulation of the cells was attempted using phorbol myristearate, 10  $\mu$ g/ml in buffer, and thrombin (IIa), 10 U/ml.

#### Immunoisolation of radiolabelled Factor V:

$^{35}\text{S}$ -methionine (1300 Ci/mmol) was obtained from Amersham. Glycine-quenched CNBr-activated Sepharose, burro anti-bovine IgG-Sepharose, anti-ATIII-Sepharose and mouse monoclonal anti-bovine factor V/Va IgG Sepharose were prepared as per March et al. (47) using Sepharose CL-4B from Pharmacia Fine Chemicals, Uppsala, Sweden. Buffer used in the procedure was 0.02 M Tris-HCl, 0.15 M NaCl, 5 mM  $\text{CaCl}_2$ , pH 7.2. Redi-Solv HP, from Beckman, was used as scintifluor. All other materials were reagent grade.

#### $^3\text{H}$ -Leucine:

The hypothesis maintains that endothelium synthesizes and secretes Factor V, to furnish proof for this claim, the Factor V thus produced had to be isolated and identified. To distinguish Factor V present in the serum of the

medium used to maintain the cells from intrinsically produced Factor V,  $^3\text{H}$ -leucine was added to the cultures in medium 199 in the initial isolation procedures.  $^3\text{H}$ -leucine was chosen, because the Factor V molecule has 240 leucine residues (32). After a four-five day incubation period, the culture supernatant was collected and applied to burro anti-bovine Factor V IgG-Sepharose, which had been prepared by the method of March et al. (47). The supernatant was chromatographed at room temperature, with wash buffer, 0.02 M  $\text{NaPO}_4$ , 0.15 M NaCl, pH 7.4, applied at 0.5 mL/minute. After application of the radiolabelled supernatant, the column was washed with buffer, 2 mL fractions were collected, and fractions were monitored by scintillation counting. When the buffer wash fractions had scintillation counts of 100 cpm or less, the resin was eluted using 1 mL of 8 M Urea. One milliliter fractions were collected, and scintillation counted to determine fractions with highest radioactivity. These peak fractions were dialyzed vs. 0.2 M acetic acid, with one change, overnight. Polyacrylamide gel electrophoresis was accomplished preparatory for autoradiography. Because  $^3\text{H}$  is such a low beta emitter, and recovery was so small, the use of  $^3\text{H}$  was abandoned.

#### $^{35}\text{S}$ -Methionine:

Since  $^{35}\text{S}$  is a stronger beta-emitter than  $^3\text{H}$ ,  $^{35}\text{S}$ -methionine was added to methionine-free RPMI 1640 (from GIBCO's Select-an-Amine kit) for incorporation into products synthesized by the cultured cells. Cells were cultured on 100mm Co-Star plastic tissue culture dishes, and allowed to reach confluence in medium 199. The medium was changed to methionine-free RPMI 1640 (from "Select-an-Amine" medium kit, Gibco, Grand Island, N.Y.), supplemented with

10% FBS and pen-strep, then 0.5 mCi  $^{35}\text{S}$ -methionine (1300 Ci/mmol, Amersham) was added to each of four 100mm culture dishes of confluent cells. Culture  $^{35}\text{S}$ -supernatant was collected from the four dishes (10 ml/dish) after 4-5 days, and pre-chromatographed on glycine-quenched, CNBr-activated Sepharose, prepared as per March *et.al.* (47). A 50 ul sample of the flow-through  $^{35}\text{S}$ -supernatant was bioassayed, to ensure Factor V/Va activity was present. The pre-chromatographed  $^{35}\text{S}$ -supernatant was divided equally into four aliquots (approximately 10 ml each). Immunoisolation chromatography was performed, using the aforementioned mouse monoclonal anti-bovine Factor V/Va IgG (IB6), which had been immobilized on CNBr-activated Sepharose and provided by Dr. W.B.Foster (36). Mouse monoclonal anti-human antithrombin III (ATIII)-Sepharose was generously provided by Dr. M.E. Nesheim (Mayo Clinic, MN) for use as a control. The antibody resins were equilibrated with 0.02 M tris-HCl, 0.15 M NaCl, 5 mM  $\text{CaCl}_2$ , pH 7.2 (column buffer). The equilibrated resins (2-0.5 ml aliquots of each resin) were then placed into four 15 ml polystyrene conical centrifuge tubes (which had been pre-coated with unused FBS supplemented medium, then rinsed with column buffer), and the  $^{35}\text{S}$ -supernatant aliquots added. The tubes were tightly closed, and placed on a rocker platform for 30-45 minutes. Resin- $^{35}\text{S}$ -supernatants were poured into four glass pasteur pipette columns with glass wool frits, and  $^{35}\text{S}$ -supernatant flow through was bioassayed to determine Factor V content. At this point, one anti-Factor V-Sepharose column and one anti-ATIII-Sepharose column were each treated with 10 U of bovine thrombin in one milliliter of column buffer. All columns were washed with column buffer, and radioactivity in the 2 ml fractions was followed by monitoring scintillation counts in a Beckman LS-7500

liquid scintillation counter, with Beckman Redi-Solv HP as the scintifluor. When counts were less than 100 cpm, elution of bound counts was accomplished by using 0.2 M glycine, pH 2.8. Elution fractions were monitored via scintillation counts. The two peak fractions (2 ml each) were dialyzed vs. 2-800 ml volumes of 0.2 M acetic acid over night with one change of dialysate. All eluants were then lyophilized in preparation for electrophoretic analysis.

#### Electrophoresis/Autoradiography:

Acrylamide, N,N'-methylenebisacrylamide, ammonium persulfate, TEMED (N,N,N,N-tetramethylethylenediamine), and the Protean apparatus were obtained from Bio-Rad. Upper electrode buffer was 0.1% sodium dodecyl sulfate (SDS), 0.04 M Tris borate, pH 8.6, and lower electrode buffer was 0.04 M Tris borate, pH 8.6. Gradient slab gels were made as follows for a 5—15% gradient: 5% = 0.1 parts 47.5% acrylamide:2.5% bis-acrylamide; 0.25 parts 0.0308 N HCl, 0.4244 M Tris, pH 9.18 (lower gel buffer); 0.01 part 10% SDS; 0.605 parts H<sub>2</sub>O; 0.015 parts 0.005% riboflavin; 0.01 parts 1% TEMED (v/v); 0.01 parts 10% ammonium persulfate (w/v), and 15% = 0.3 parts 47.5% acrylamide:2.5% bis-acrylamide; 0.25 parts lower gel buffer; 0.25 parts 40% sucrose (w/v); 0.01 parts 10% SDS; 0.16 parts H<sub>2</sub>O; 0.015 parts 0.005% riboflavin; 0.01 parts 1% TEMED; 0.005 parts 1% ammonium persulfate. Composition of the 4% stacking gel was 0.25 parts 12.8% acrylamide:3.2% bis-acrylamide, 0.25 parts 0.0267 H<sub>2</sub>SO<sub>4</sub>, 0.0541 M Tris, pH 6.14, 0.25 parts 40% sucrose, 0.005 parts 10% TEMED (v/v), 0.001 parts 10% SDS, 0.24 parts H<sub>2</sub>O, 0.005 parts 10% ammonium persulfate. EnHance was obtained from New England Nuclear. Kodak XAR-5 x-ray film was obtained from Eastman Kodak, Chicago, IL.

Lyophilized  $^{35}\text{S}$ -samples were dissolved in Neville sample preparation buffer (48), as had the earlier  $^3\text{H}$ -leucine eluant samples, (10% SDS and 2% B-mercaptoethanol) plus 2.5% 0.5 M  $\text{Na}_2\text{EDTA}$ .  $^{125}\text{I}$ -bovine Factor V and thrombin-activated  $^{125}\text{I}$ -bovine Factor Va were used as standards. Samples were heated at  $90^\circ\text{C}$  in a heat block for 10-15 minutes, and equivalent quantities of cpm were added to each well of a Neville 5—15% gradient polyacrylamide slab gel with a 4% acrylamide stacking gel (48), and electrophoresed in a Biorad "Protean" electrophoresis apparatus. Following electrophoresis, the gels were fixed in 100% trichloroacetic acid-glacial acetic acid-methanol-water (1:1:3:5) (v:v) for 30 minutes, then fixed in "EnHance" (New England Nuclear) for 45 minutes, followed by water rehydration for at least 45 minutes. The fixed gels were mounted on electrophoresis paper, and dried on a slab gel drier. The dried gel was placed inside a Wafer Rigid-Form x-ray cassette, lined with DuPont "Lightning Plus" intensifying screens, with Kodak XAR-5 x-ray film for exposure at  $-70^\circ\text{C}$ , with time of exposure dependent upon cpm of sample applied to gel. X-ray film was developed in a Kodak "X-omat" automatic film processor.

## RESULTS:

### Tissue Culture:

There was a great variability among cells harvested from aorta to aorta. However, most grew well in culture when plated at 30-50% cell density. Density less than 30% yielded cultures with initial atypical morphology, and decreased culture viability. Collagenase digestion of cells from the aortic lumen yielded clumps of 10-100 cells; therefore, an accurate count of the number of cells plated per dish was difficult; so, equivalent quantities of cell suspensions were added to each culture container. An obvious seasonal trend for culture viability existed, with each season change, and summer, providing the least viable, and most contaminant susceptible cultures. From a subjective viewpoint, cultures remained healthier (i.e., retained morphological characteristics) when culture medium was changed at 3-4 day intervals. At the previously mentioned plating density, cultures were usually confluent within 5-7 days.

BAE cells were versatile where culture surfaces were concerned, and were cultured in a variety of containers and surfaces. Cells grew well on the Cytodex 3 microcarriers, rapidly attaching to the collagen pre-coating. Cuvettes provided a challenge at first, with many failures, until the method of gas-sterilizing cuvettes and placing them into quadriplexes inside Co-Star Cluster 6 wells was used. The most difficult surface was provided by glass cover slips. Endothelial cells grew on glass, but processing them for fluorescence often removed huge sheets of cells. Fibronectin pre-coating of the glass, coupled with very delicate washing and pipetting of reagents onto the cover

slip-cell surface, alleviated the problem. As long as complete medium pre-conditioned the surfaces, presumably leaving a fibronectin coating (from the FBS), cells would adhere to almost any cell culture cleaned surface.

#### Bioassay and RIA:

Bioassay results revealed a linearly increasing level of factor V activity in the daily samples of BAE culture supernatant. However, the bioassay failed to detect active factor Va in the supernatant. Radioimmunoassay data consistently showed an increasing level of factor V antigen in the medium, which was much higher than the bioactivity detected by the bioassay. In Figure 9, a comparison of results from the two assay methods on culture supernatant taken from identical samples of a representative confluent culture series over a four day period between medium changes, is shown. Both bioassay and RIA indicate an increase in factor V activity/antigen levels over the four day period. Additional radioimmunoassay results are shown in Figure 10 from a separate culture, also covering a four day period. To ensure that levels of factor V activity/antigen detected were not due to the heat-inactivated fetal bovine serum supplement in the medium, samples of unused FBS supplemented medium were used as background and were subsequently subtracted from the raw data prior to plotting. Considering the lability of factor V, heat inactivation of the FBS may have been responsible for the lack of factor V activity in the bioassay of unused medium. The differences between bioassay and RIA results may be attributable to the fragmentation of the labile factor V molecule in the culture supernatant, as later evidenced by results from the immunoisolation. In addition, triton-solubilized cells also failed to display factor V activity in the

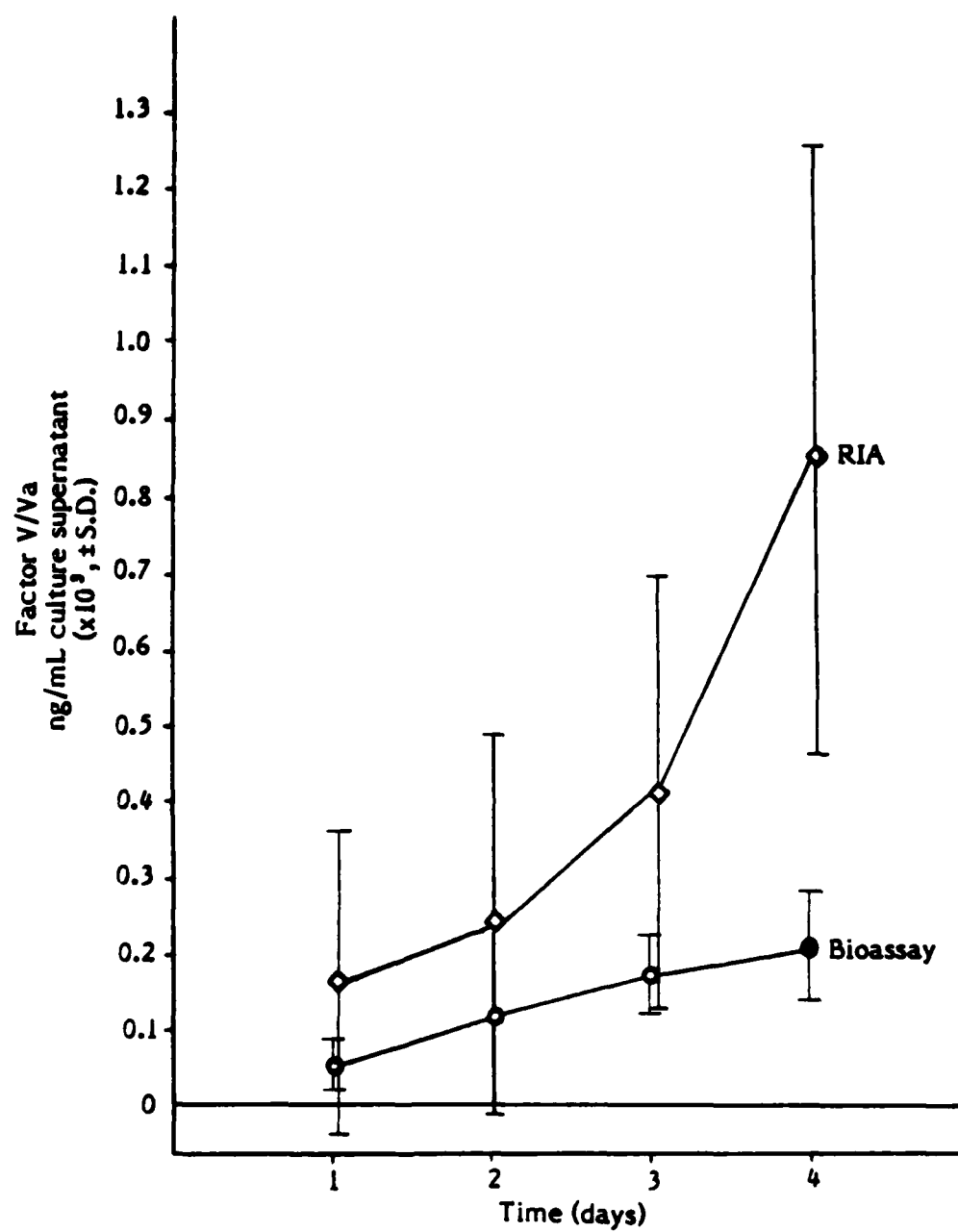


Figure 9



Figure 9. Factor V Production by Confluent Cultures of Bovine Aortic Endothelial Cells. On day 0 confluent cultures were provided fresh medium. Over the next four days, daily supernatant medium samples were taken, immediately analyzed for functional factor V/Va (bioassay) and the remainder of the day's sample stored at  $-20^{\circ}\text{C}$  to be analyzed for factor V-reactive protein (RIA). The error bars at each point represent the mean and standard deviation for four separate cultures. The polyclonal nature of the burro antbovine factor V IgG resulted in the detection of all fragments of factor V. The divergence of RIA and bioassay data over the four day time course most likely is the result of degradation of factor V following synthesis.

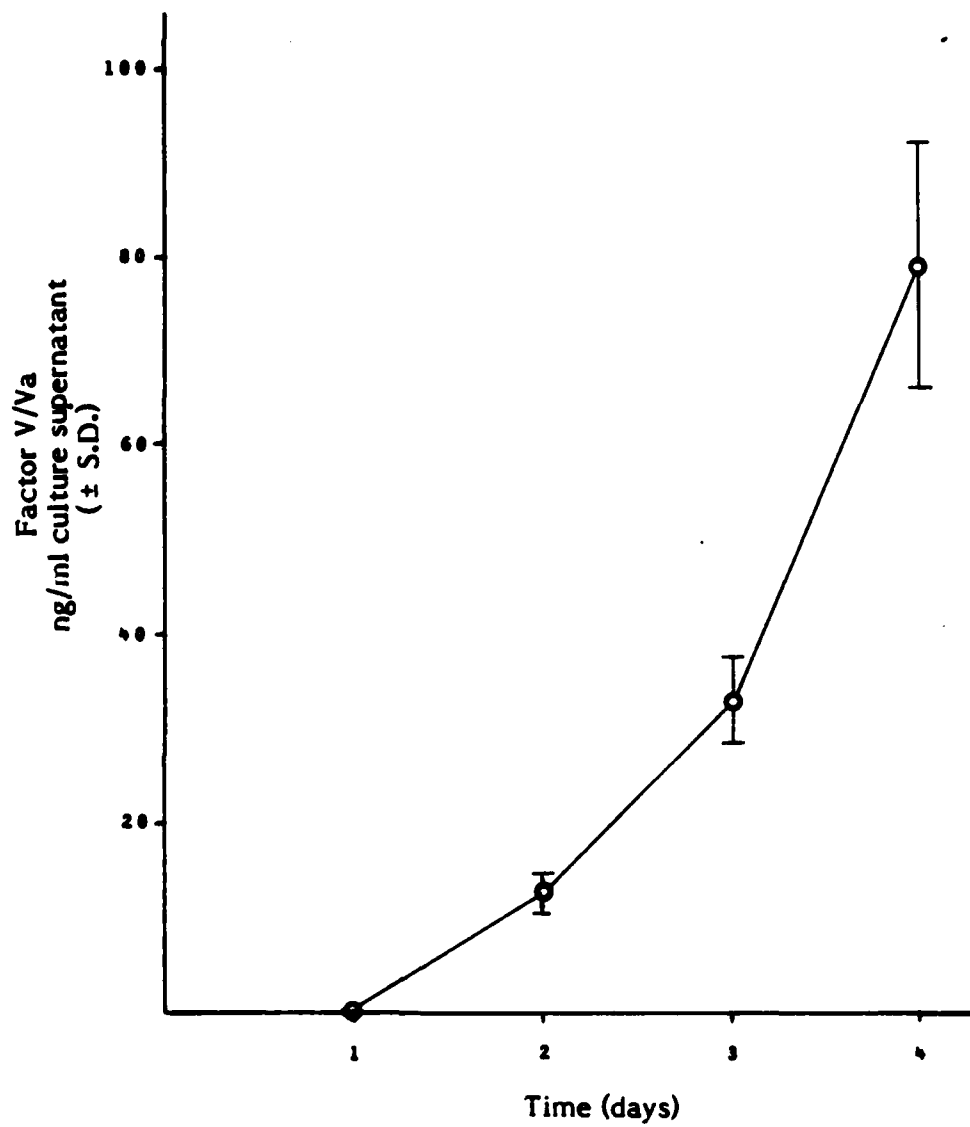


Figure 10

Figure 10. Factor V Production by BAE Cultures. Radioimmunoassay data of samples taken from confluent cultures, in the same manner as in figure 9, indicating lower levels of factor V production than the culture series shown in figure 9. RIA results were peculiar to the particular culture being analyzed and factor V levels varied over a wide range from culture to culture.

bioassay, but a constant low antigen level (20-50 ng/ml) was detectible with the RIA. Triton solubilization has been shown by Tracy et al. (42-43) not to interfere with either the bioassay or the RIA results.

Some very interesting RIA results from one human umbilical vein culture, grown to the same stage of confluence as tested BAE cultures, indicated factor V production by human endothelial cells, but at much lower levels than BAE cells. An interesting note: triton solubilized human cells resulted in greater antigen levels than in 3 day culture supernatant; whereas, similarly treated bovine cells revealed factor V/Va antigen levels which were barely detectible by the RIA. However, a five day culture supernatant sample indicated factor V antigen at a 30% greater level than had the solubilized cells (see Figure 11). Bioactivity was undetectible in either the solubilized cells, or three day culture supernatant. Bioassay was not accomplished on the five day supernatant (reagent nonavailability).

Bioassay and RIA provided a quantitative determination of factor V/Va present in the sample tested. This quantitation was incongruous, in that activity, as determined by the bioassay, did not equal antigenicity, as determined by the radioimmunoassay. The difference is most likely explained by defining activity and antigenicity. Activity is ascribed to the part(s) of factor V which participate in the physiological response to a hemostatic insult, as previously described in introduction. Whereas, antigenicity corresponds to any domain of the factor V molecule which has raised an immune response that resulted in the polyclonal antibody used in the RIA. Only a small portion of the factor V molecule (74K and 94K thrombin cleavage products) is bioactive, while all fragments, as well as the intact molecule, may provide an antigen.

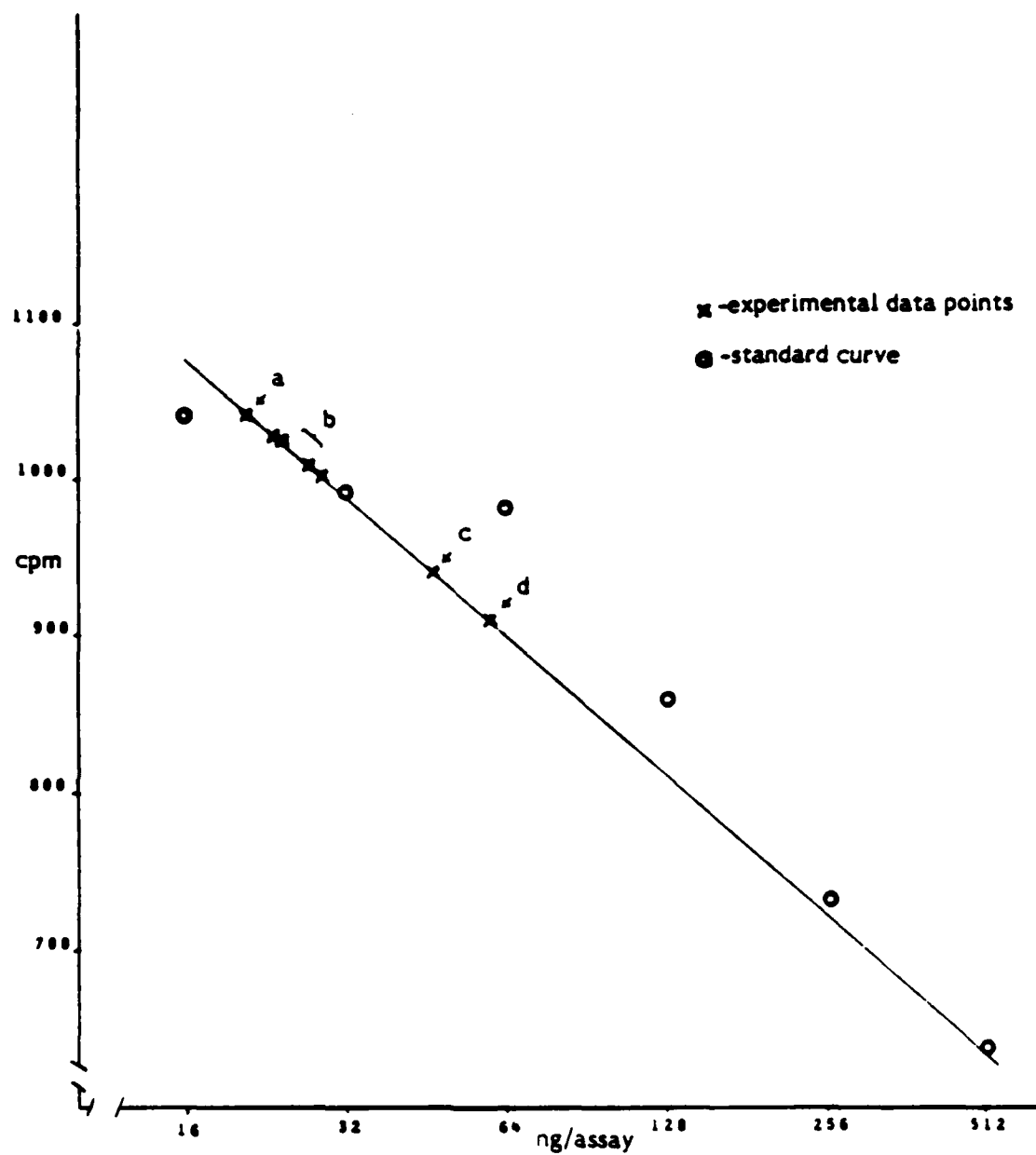


Figure 11

Figure 11. Factor V Production by Human Umbilical Vein Endothelial (HUVE) Cultures. Radioimmunoassay was accomplished on culture supernatant samples from one successful HUVE culture ( a = unused medium; b = four separate three day cultures; c = tritonized cells; d = five day culture (from b, with all four supernatants combined)). The standard curve, upon which the experimental data points are plotted, was expanded from the original plot to show only the range wherein the experimental data occurred.

Bioassay results varied greatly from culture to culture. Some cultures produced activity at very low levels in the supernatant, requiring virtually straight samples in the assay, while other culture supernatants had to be diluted 1:5 for assay determination. After cells had been in culture for 3-4 days, only factor V activity could be detected. In some unused culture medium, occasionally an initial low level of factor Va could be detected at the lower limits of the bioassay's sensitivity. This factor Va declined rapidly after culture feeding, and became undetectable within two days following feeding, while a concomitant increase in factor V levels occurred. Factor V levels continued to increase and accumulate, to much higher levels than initial factor Va detection, thus implying that this activity/antigenicity was produced by the cells. In some of the experiments, immunoinhibition studies were conducted using both polyclonal and a combination of inhibitory and noninhibitory monoclonal antibodies. In these experiments culture supernatants were assayed for factor V bioactivity using human factor V-deficient plasma. A typical experiment is presented in Table 2. For this experiment, factor V/Va was determined by bioassay to be present at a concentration of 133 ng/ml. Incubation of the cell supernatant with the inhibitory monoclonal antibodies, IIC3 and IVC6, resulted in total loss of the observed bioactivity. In contrast, incubation with a noninhibitory anti-factor V monoclonal antibody (IB6) led to no inhibition of bioactivity. A similar result was obtained with nonimmune burro serum was compared to immune burro anti-factor V IgG. The latter totally abolished factor V bioactivity, whereas the former had no effect.

**Table 2. Immunoinhibition of Factor V Bioactivity**

Antibody Added	Specificity	Factor V/Va Recovered (mg/ml)	Percent Recovery
None	—	133	100
IIC3*	Inhibitory $\alpha E^\dagger$	0	0
IVC6*	Inhibitory $\alpha E^\dagger$	0	0
IB6*	Noninhibitory $\alpha D^\dagger$	115	86
Burro $\alpha V_s$	—	0	0
Nonimmune Burro	—	118	88

\* To 90  $\mu$ l culture fluid supernatant, 10  $\mu$ l of a 0.5 mg/ml antibody solution was added.

† The factor Va chain specificity of the antibody is specified.

s To 90  $\mu$ l culture fluid supernatant, 10  $\mu$ l of a 10 mg/ml antibody solution was added.



### Immunofluorescence:

The increase of factor V in cell culture supernatants, as determined by RIA, indicated that factor V/Va antigen was present in the cultures. In order to determine if this antigen was associated with the cells (either in or on), immunofluorescence was undertaken.

Immunofluorescence was accomplished on BAE cultures grown on fibronectin coated glass coverslips using both murine monoclonal and burro polyclonal antibodies to bovine factor V/Va. Although positive immunofluorescence results were obtained with both the monoclonal and polyclonal reagents, the polyclonal reagents gave rise to much greater intensity of fluorescence. Factor V antigen immunofluorescence activity varied with the age of the culture, with optimum fluorescence attained when the culture was 2-3 days post-confluent. Even at this optimum, not all cells were immunofluorescence positive.

The intensity of fluorescence observed with monoclonal antibody staining was distinctly positive, but weak. For the monoclonal reagents, the fluorescence appeared to be diffuse and restricted to the cytoplasm of selected cells, perhaps those at a particular stage of cell cycle (Figure 12a). Negative controls prepared using nonimmune IgG, at the same concentration as the monoclonal antibody, and photographed with the same exposure time, did not show fluorescence (Figure 12b).

In contrast to the weak immunofluorescence observed with monoclonal reagents, relatively high fluorescence intensity was observed when the polyclonal reagents were used in the immunofluorescence experiments. Figure 13a represents the immunofluorescence of cells treated with burro anti-factor V antibody and counterstained with fluorescein labelled goat anti-horse serum.

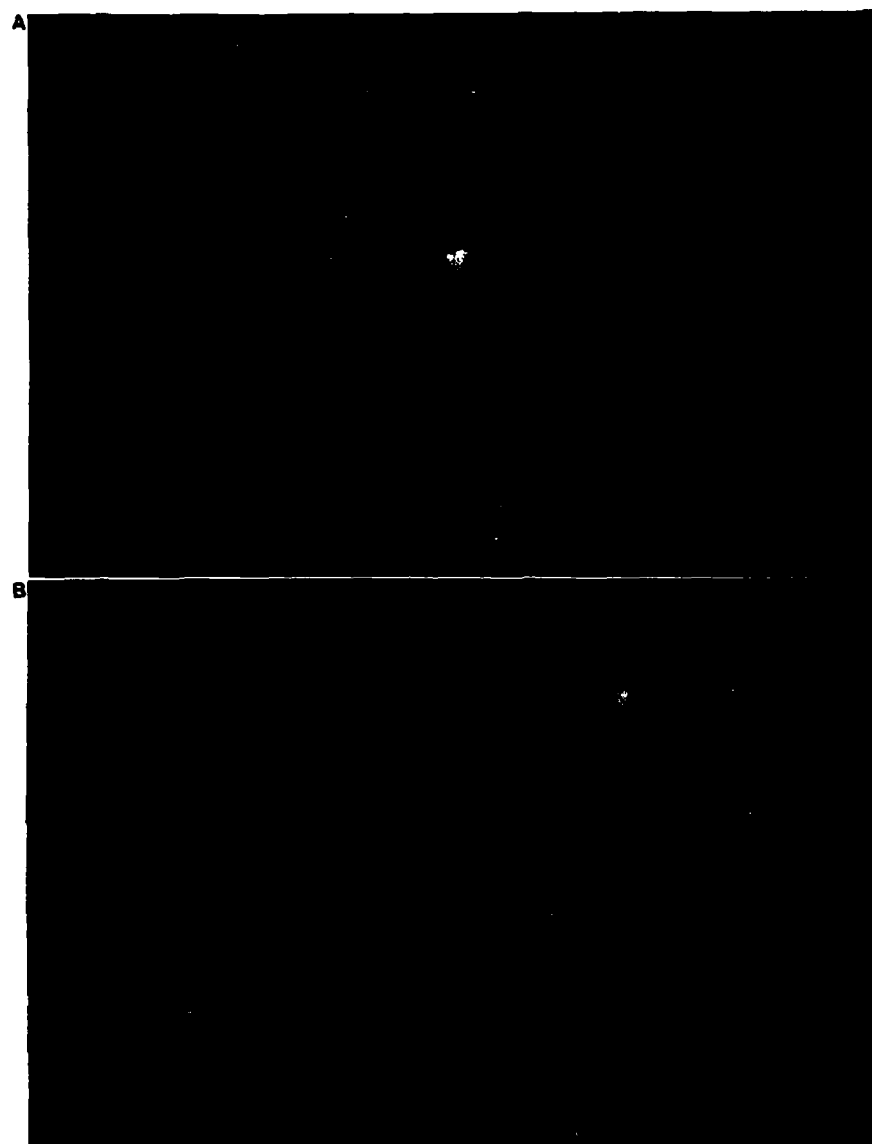


Figure 12

**Figure 12. Indirect Immunofluorescence of Factor V/Va Associated with Bovine Aortic Endothelium (BAE). Confluent cultures of BAE were fixed in cold buffered formalin-acetone prior to indirect immunofluorescence processing. Primary antibodies were A) murine monoclonal antbovine Factor V/Va (IB6) and B) nonimmune mouse IgG, both were applied at equivalent concentrations. The secondary antibody, for both A and B, was a FITC-conjugated goat antimouse IgG, which produced the resultant fluorescence. Factor V/Va, associated with BAE in culture, is apparent from the fluorescence observed in A. (Magnification 480x)**

The nonimmune control is presented in Figure 13b. For the results represented in Figure 13a, the experiment was conducted directly in a 35 mm culture dish, and fluorescence observed by epi-illumination fluorescence microscopy. Distinct perinuclear cytoplasmic staining is observed in some, but not all, cells, a result alluded to in the discussion of monoclonal results. Furthermore, various gradations of immunofluorescent staining were observed, suggesting that factor V synthesis by endothelial cells in culture is not a continuous property for all endothelial cells, but may be subject to regulation of some sort, perhaps associated with cell cycle.

To provide a positive control, duplicate BAE cells were processed, at the same time, for immunofluorescence, using polyclonal Behring rabbit anti-human factor VIII:Ag/vWF IgG. In Figure 14a, the resulting immunofluorescence was displayed by large, bright, white-green granules covering the entire microscopic visual field, both associated with cells and a strandy matrix-like material (possibly fibronectin or other basal lamina associated materials which endothelial cells are known to produce in culture). This positive immunofluorescence for factor VIII:Ag/vWF is used as one of the criteria for identification of endothelium (20,49,50).

As negative controls, non-immune rabbit IgG, non-immune mouse IgG and non-immune burro sera were used. The non-immune results provided a dull diffuse background, which appeared several orders of magnitude less intensely fluorescent than had the anti-factor V/Va or anti-factor VIII:vWF antibodies, thus providing a low contrast background for comparison to the positive antibodies (see Figures 12b, 13b, and 14b). While the immunofluorescence data, indicating that factor V/Va is in/on endothelial cells in culture, is consistent

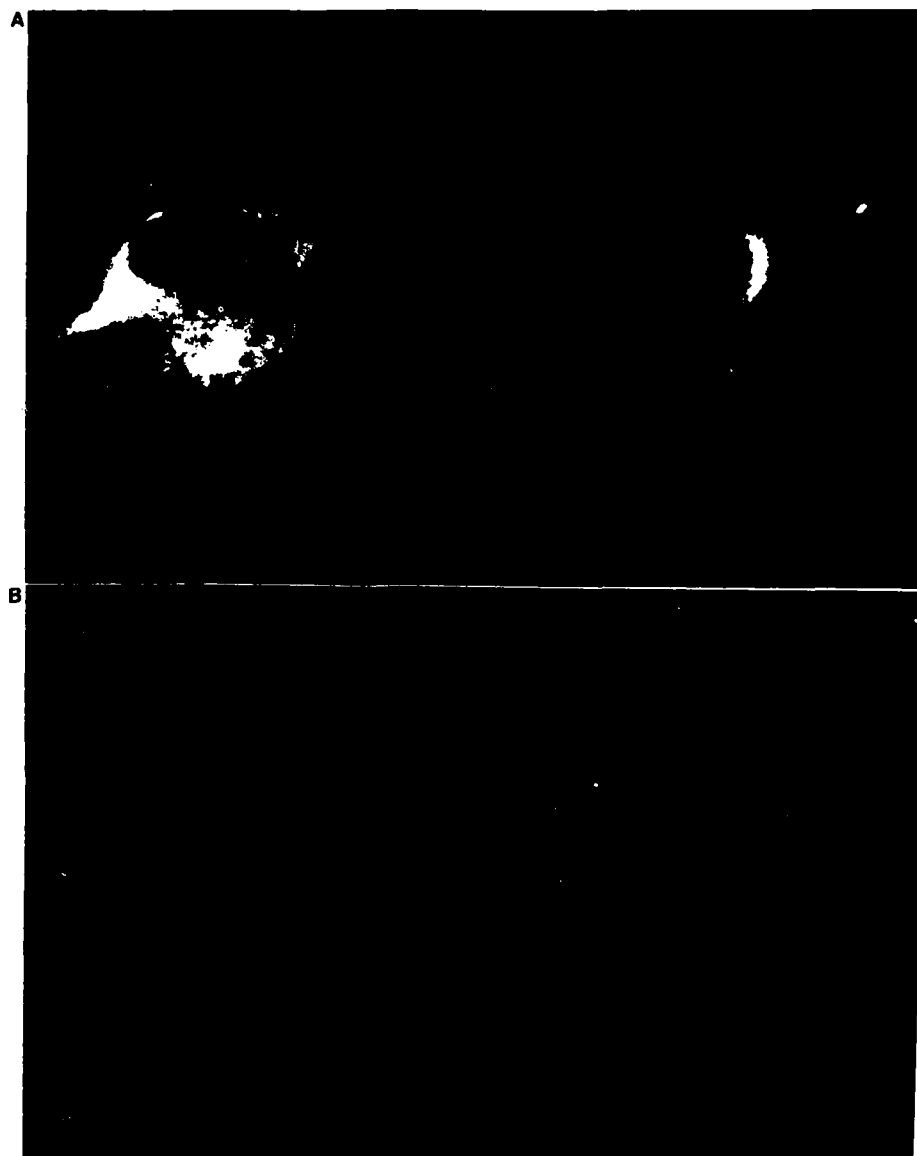


Figure 13

**Figure 13. Indirect Immunofluorescence of Factor V/Va Associated with Bovine Aortic Endothelium (BAE). Preconfluent cultures of first passage BAE were fixed in 70% ethanol in 35mm plastic dishes and analyzed with A) 1:250 dilution of burro antibovine Factor V/Va IgG (10 mg/mL) diluted in a 1:250 dilution of nonimmune burro serum in 1% bovine serum albumin, and B) the nonimmune burro diluent. The secondary antibody for both A and B was goat antihorse IgG conjugated with FITC and diluted 1:200 in PBS-1% BSA. (Magnification 800x)**

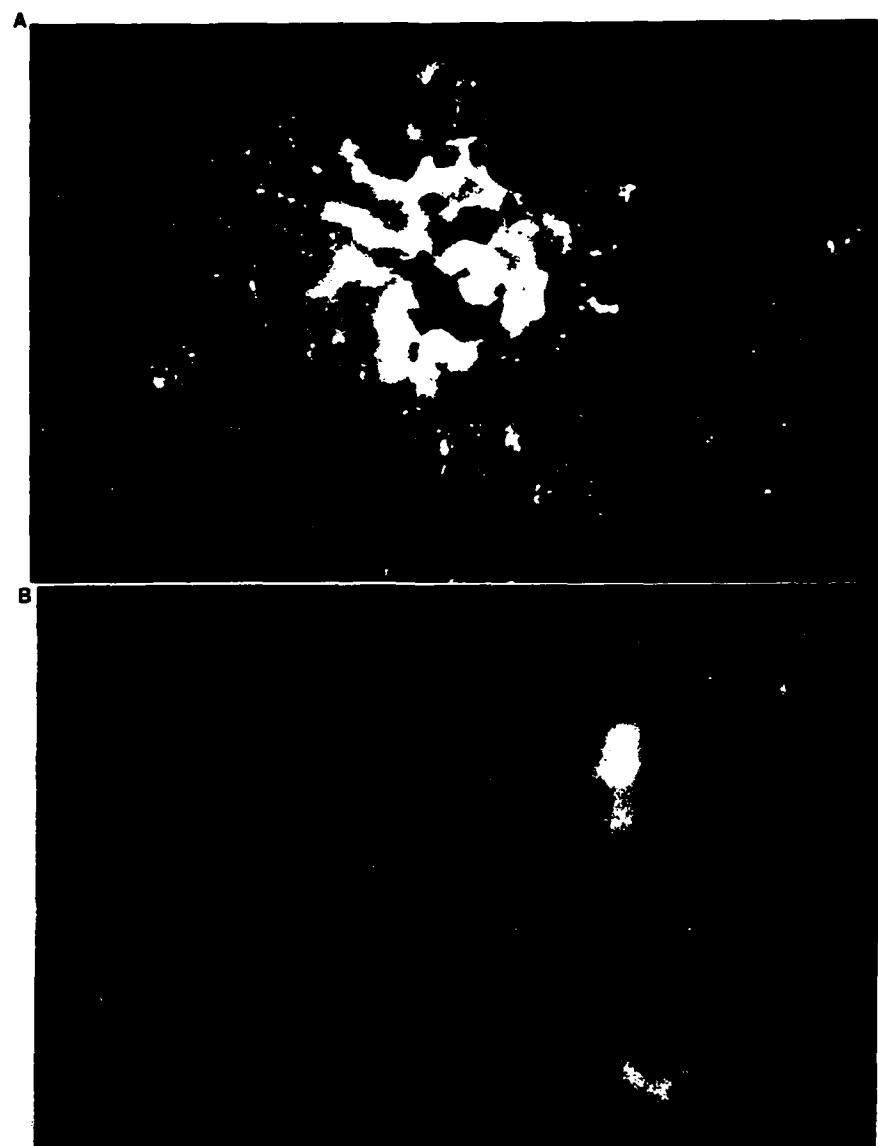


Figure 14

Figure 14. Indirect Immunofluorescence of Factor VIII:vWF Associated with Bovine Aortic Endothelium (BAE). As a positive control for the Factor V immunofluorescence, cells from the same cultures as Figure 2 were processed for indirect immunofluorescence with primary antibody A) Behring rabbit anti-human factor VIII:vWF, and B) nonimmune rabbit IgG, both were applied at equivalent concentrations. The secondary antibody was FITC-conjugated goat antirabbit IgG, which produced the resultant fluorescence. (Magnification 480x)



with RIA and bioassay results, it does not provide conclusive evidence concerning synthesis by the endothelial cell.

In some of the immunofluorescence studies, cells were treated with 1 U thrombin (IIa) both before and after fixation. The IB6 IgG functioned best with non-thrombin treated cells, while IIIA5 functioned best with thrombin treated cells, though both revealed immunofluorescence irregardless of thrombin treatment of the cells. Thrombin treatment had no observable effect on immunofluorescence by Behring antiserum.

#### Factor V Synthesis by Endothelium:

Since factor V was predicted to be less than one percent of the proteins synthesized by endothelial cells, difficulty in isolating the product was expected. Tritium-labelling proved to be an inadequate method of detection of factor V, even though there are many leucine residues in the factor V molecule. Several immunochromatography isolations were accomplished, revealing so many protein bands on the autoradiograms, that any factor V, which may have been present, was heavily masked. To try to increase the detection of factor V on autoradiograms,  $^{35}\text{S}$ -methionine was used as the amino acid to be incorporated into protein synthesis. A decrease in protein products was expected, since fewer proteins contain methionine than leucine. The following is representative of results from  $^{35}\text{S}$ -methionine incorporation.

The starting material (4-5 day post-confluent culture supernatant) had  $1.57 \times 10^8$  cpm/10 ml, as well as Factor V activity by the bioassay (27 ng/ml). The flow-through from the glycine-Sepharose column was bioassayed to ensure that Factor V activity was present prior to immunoisolation chromatography.

No Factor V activity was lost, in fact, bioactivity, as determined by the bioassay, increased slightly (32 ng/ml). This glycine-Sepharose pre-chromatography step was included in the protocol to decrease the amount of non-specific binding by the  $^{35}\text{S}$ -labelled products, which were binding to the immunoabsorbant-Sepharose. This non-specific binding had presented a problem in recovery. The pre-chromatography flow-through retained  $1.48 \times 10^8$  cpm/10 ml, which was applied to the anti-Factor V-Sepharose and anti-ATIII-Sepharose columns. The anti-ATIII-Sepharose was chosen as a control, solely on the grounds that the ATIII antigen mass is entirely outside the molecular weight range of Factor V/Va antigens. Whether, or not, ATIII is produced by endothelial cells is unknown at this time.

Bioassay of the flow through from the anti-Factor V-Sepharose indicated an undetectable level of bioactivity, which implied that Factor V activity was retained by the resin. Bioassay of the anti-ATIII-Sepharose flow-through revealed an increase in Factor V activity (43 ng/ml), implying that Factor V had not been retained by this resin, and that, potentially, some depletion of inhibitor(s) had occurred. At this point 10 U of thrombin were added to one of each of the pairs of antibody-Sepharose columns. The activation of the proposed Factor V molecule, while immobilized on the anti-Factor V-Sepharose, provided significant evidence for identification of the eluted  $^{35}\text{S}$ -labelled substance as Factor V, while thrombin treatment of the anti-ATIII-Sepharose served as a control for this identification. Tris was used in the buffers, because it does not interact with calcium, and it is compatible with the antibody-Sepharose resin. Fractions were monitored by following the  $^{35}\text{S}$ -isotope content. Following elution of the unbound radioactivity, the columns were

washed with 2M NaCl, to further decrease non-specific binding of  $^{35}\text{S}$ -labelled protein. This high salt wash was followed by column buffer wash to background cpm. Since elution of bound radioactivity was accomplished at low pH, Factor V presence could not be monitored by bioactivity; therefore, elution was monitored by  $^{35}\text{S}$  content. The glycine-eluted  $^{35}\text{S}$ -products came off discretely in the first 2-3 fractions, with the anti-Factor V-Sepharose yielding  $8 \times 10^4$  total cpm in the peak fraction, and the anti-ATIII-Sepharose yielding  $4.3 \times 10^4$  total cpm in its peak fraction. See chromatogram, Figure 15.

#### Autoradiography

Figure 16 is an autoradiogram of eluants from the anti-Factor V/Va-Sepharose with  $^{125}\text{I}$ -bovine FV and  $^{125}\text{I}$ -bovine Va standards. The anti-Factor V-Sepharose  $^{35}\text{S}$ -eluant from the non-thrombin treated column in lane b has a  $^{35}\text{S}$ -labelled peptide band at 330K molecular weight, corresponding to the adjacent  $^{125}\text{I}$ -Factor V standard in lane c. While the control anti-ATIII-Sepharose  $^{35}\text{S}$ -eluant in lane d has a marked absence of bands in the region where Factor V related products occur. A very small fraction of total  $^{35}\text{S}$ -labelled products, from the cell culture supernatant start material (lane a), can be accounted for by the Factor V. This small fraction made the pre-chromatography step, as well as the 2M NaCl wash, necessary to decrease the non-specific binding, so that visualization of the recovered "Factor V" would be possible on the autoradiogram. Lane e is the thrombin treated anti-Factor V-Sepharose  $^{35}\text{S}$ -eluant, and has peptide bands at 94K and 74K molecular weight, corresponding to the thrombin-treated (activated)  $^{125}\text{I}$ -Factor Va standard in lane f. The 74K molecular weight peptide of the  $^{125}\text{I}$ -Factor Va standard in

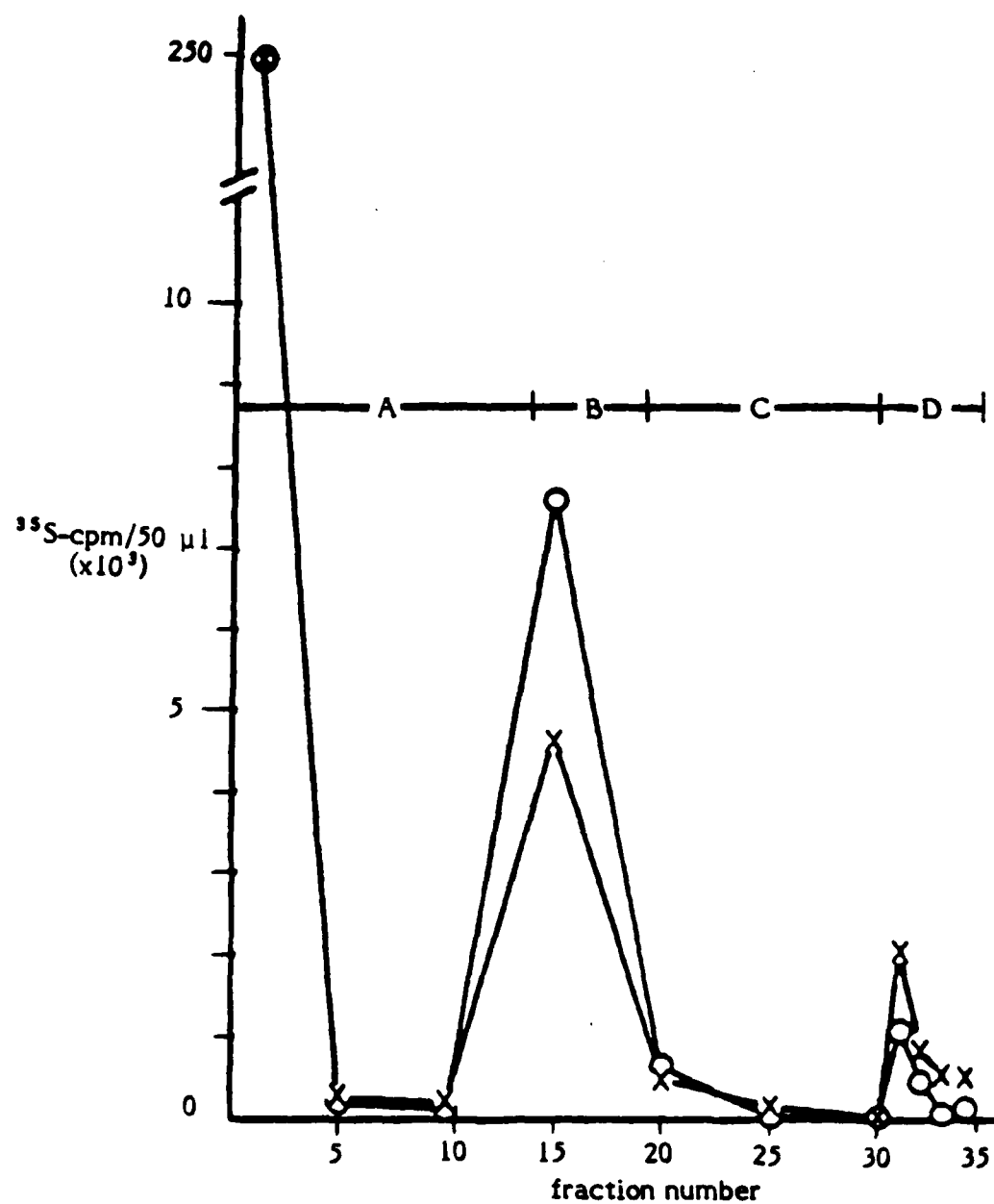


Figure 15

Figure 15. Chromatography of Radiolabelled Proteins Using Immunoabsorbant Sepharose Columns. Culture supernatant medium was collected from confluent BAE cultures, which had been incubated with  $^{35}\text{S}$ -methionine, and pretreated on glycine-Sepharose to remove major synthetic products derived from the cells. The flow-through fractions from the glycine-Sepharose prechromatography were applied to either murine antiovine factor V/Va (IB6)-Sepharose (x) or murine antihuman ATIII-Sepharose (o). The columns were washed with solutions A, B, and C and eluted with solution D. A and C = 20 mM tris, 0.15 M NaCl, 5 mM  $\text{CaCl}_2$ , pH 7.4; B = 2 M NaCl; D = 0.2 M glycine, 0.15 M NaCl, pH 2.9. Fraction volume = 2 mL.

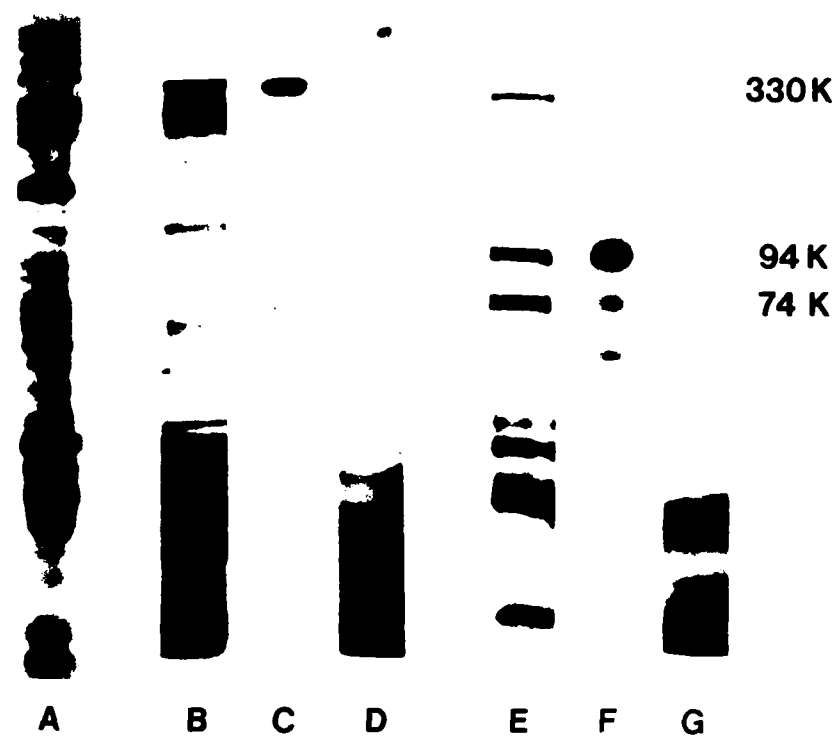


Figure 16

Figure 16. SDS gel autoradiogram of  $^{35}\text{S}$ -methionine labelled endothelial synthetic products isolated on antibody columns. Lane A corresponds to the fraction obtained after pre-treatment of the culture supernatant medium with glycine-Sepharose to remove major high molecular weight synthetic products. This supernatant-flow through was applied to the antibody columns. Lane B represents the material eluted in peak D (Fig. 15) from the murine antiovine factor V/Va antibody column. Lane C is an  $^{125}\text{I}$ -Factor V standard. Lane D is peak D (Fig. 15) from the murine antihuman ATIII antibody column. Factor V, as well as degradation products obtained from single chain factor V, are apparent in Lane B and are absent in Lane D. Lane E represents the protein eluted (peak D) from murine antiovine factor V/Va-Sepharose after treatment of bound protein with thrombin. Lane F is an  $^{125}\text{I}$ -Factor Va standard. Lane G represents peak D as obtained from the murine antihuman ATIII column after treatment of bound protein with thrombin. The bands coincident with the two factor Va noncovalently associated chains (94K and 74K) are apparent in Lane E but absent in Lane G. These results indicate that intact and thrombin-activatable factor V is produced by bovine aortic endothelium, because it is specifically bound and subsequently eluted from murine antiovine factor V/Va-Sepharose.

lane f appears very light, because it does not label well with the Bolton-Hunter technique. The composition data for the 94K and 74K peptides, as published by Guinto and Esmon (52), show relatively equivalent amounts of methionine, which predicts the relatively equivalent labelling intensity observed in lane e. The two Factor Va peptides may result from a variety of cleavage mechanisms of Factor V. It is likely that, in addition to Factor V itself, a variety of high molecular weight fragments of Factor V, as seen in lane b, are contributing to the bands in lane e. There also appears to be some low molecular weight bands in lane e, which may correspond to noncovalently associated cleavage fragments of the 94K and 74K peptides, that remained bound to the antibody column after thrombin treatment, and correspond to inactivation products of Factor V. The thrombin-treated anti-ATIII-Sepharose  $^{35}\text{S}$ -eluant in lane g is blank in the region of the Factor V thrombin activation peptides.

#### Aorta Explant Clotting Studies:

Reagents were added, one at a time, to the fibrinogen, to ensure clot formation was not intrinsic to the reagents. An optimum clotting time was obtained using PCPS vesicles at 40  $\mu\text{M}$  in a 100  $\mu\text{L}$  reaction mixture on plastic (a multi-well culture dish) and on a glass slide. Clot formation times are as shown in Table 3. Clot formation did not occur rapidly unless all reaction components were present. In addition, clot formation time appeared dependent upon the concentration of factor Va present in the reaction mixture. Plastic and glass provided a surface for clot formation, but one upon which a clot formed more slowly than on the aortic luminal surface or in the control using PCPS vesicles on those surfaces.



Table 3. Aorta Explant Clotting Studies.

	fibrinogen	Ca <sup>++</sup>	buffer	Xa	Va	II	PCPS	time
P	+	+	+	-	-	-	-	>100s
G	+	+	+	-	-	-	-	>100s
A	+	+	+	-	-	-	-	>100s
P	+	+	+	-	-	-	+	>100s
G	+	+	+	-	-	-	+	>100s
P	+	+	+	+	-	-	+	>100s
G	+	+	+	+	-	-	+	>100s
A	+	+	+	+	-	-	-	>100s
P	+	+	+	+	+	-	+	>100s
G	+	+	+	+	+	-	+	>100s
A	+	+	+	+	+	-	-	>100s
P	+	+	+	+	+	+	-	50s
G	+	+	+	+	+	+	-	71s
A	+	+	+	+	+	+	-	39s
P	+	+	+	+	*	+	-	41s
G	+	+	+	+	*	+	-	50s
A	+	+	+	+	*	+	-	19s
P	+	+	+	+	*	+	+	19s
G	+	+	+	+	*	+	+	14s

Table 3. Aorta Explant Clotting. To determine if the endothelial lumen of the aorta provided a surface for the formation of the prothrombinase complex, a series of clot formation experiments were undertaken, with results as shown. A "+" indicates that a particular component of the prothrombinase complex was included in the reaction. The series was accomplished as indicated to ensure that fibrinogen did not have an intrinsic clotting capability. Two concentrations of factor Va were used to determine if the clot formation was dependent upon Va concentration. Though the data presented represents only three experiments, it appears that the aortic surface can be thrombogenic given the appropriate circumstances. PCPS (phosphatidyl choline-phosphatidyl serine) vesicles were used as a positive control and to provide an optimum surface for the reaction. Concentrations of reaction components were as follows: fibrinogen = 3 mg/ml;  $\text{Ca}^{++}$  = 3 mM  $\text{CaCl}_2$ ; factor Xa =  $1 \times 10^{-9}$  M; factor Va was used at two concentrations  $1 \times 10^{-9}$  (+) and  $1 \times 10^{-8}$  M (\*); prothrombin (II) = 0.1mg/ml; PCPS vesicles = 40  $\mu$  M; P = plastic surface; G = glass surface; and A = aortic surface.

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THE INTERACTION OF COAGULATION FACTOR V AND VASCULAR  
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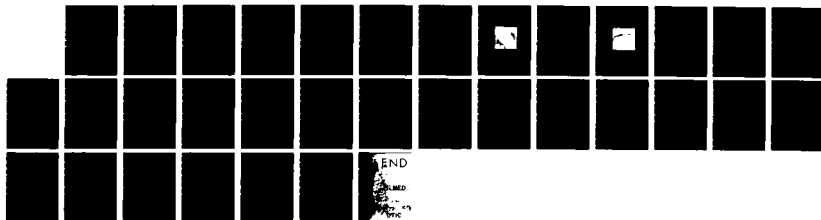
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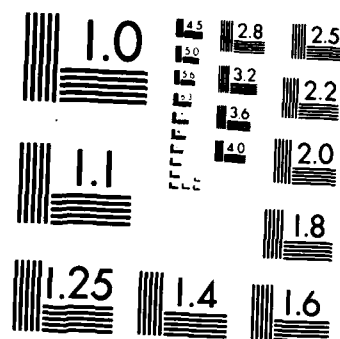
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### Factor Va Binding to Endothelial Cells:

Initial binding studies were attempted on culture grown in 24-well multi-well culture dishes. This appeared to result in a non-saturable (for  $^{125}\text{I}$ -factor Va) biphasic curve, see Figure 17. However, technical difficulties, like cells shearing from the well surface in hugh sheets, led to the alternative use of dextran microcarriers.

Cytodex III microcarriers (uniform 100 micron, collagen-coated, derivatized dextran beads) were used for these binding studies. Results obtained from binding of bovine  $^{125}\text{I}$ -factor Va to BAE cells grown on the microcarriers are in the binding curves of Figure 18. All data points were corrected for recovery and control. The number of cells per time point is as indicated. Again, there is a hint of biphasic character to the curves. Apparently, binding equilibrium occured within the first thirty minutes, under these experimental conditions. The control microcarriers also seem to provide a "surface" for prothrombinase, though definitely less than the cells. Binding was not pursued further than the data presented; therefore, definite conclusions cannot be made.

### DAPA Fluorescence Assay:

In the attempt to determine that BAE cells provide a surface for prothrombinase complex formation, DAPA assays were accomplished. At first, cells were grown on Cytodex 3 microcarries, and later, in gas-sterilized plastic cuvettes.

BAE cells grown on microcarriers did not provide a net change in fluorescence greater than that of control beads with no cells. Speculation that

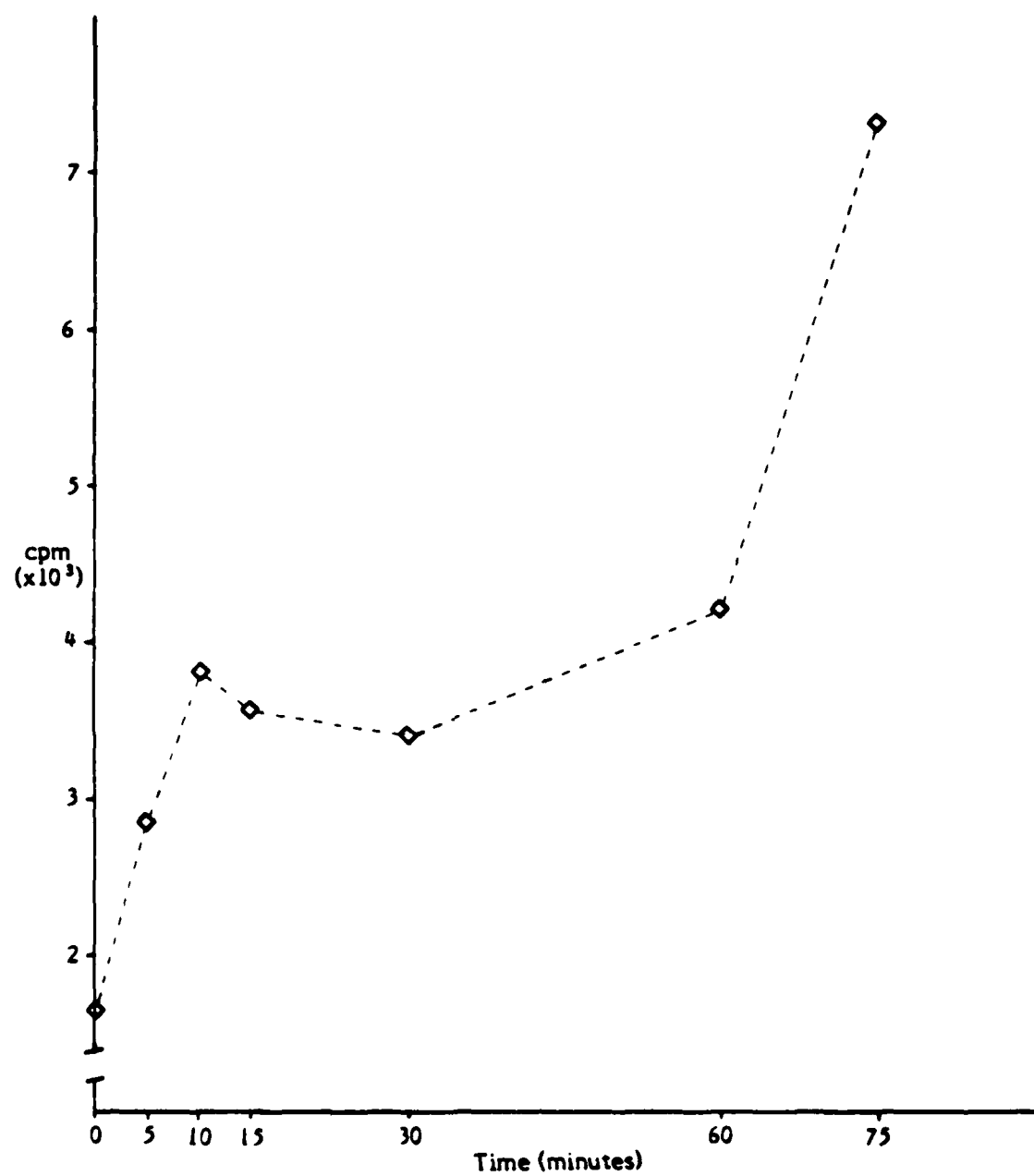


Figure 17

Figure 17. Factor Va binding to endothelial cells in multi-well culture dishes. Confluent cultures of BAE were washed three times using Earles-albumin-5 mM HEPES buffer. Then 500 ng of  $^{125}\text{I}$ -factor Va was added to each well and incubated for indicated time. Cells were solubilized with 1 M NaOH prior to samples being taken for gamma count determination.

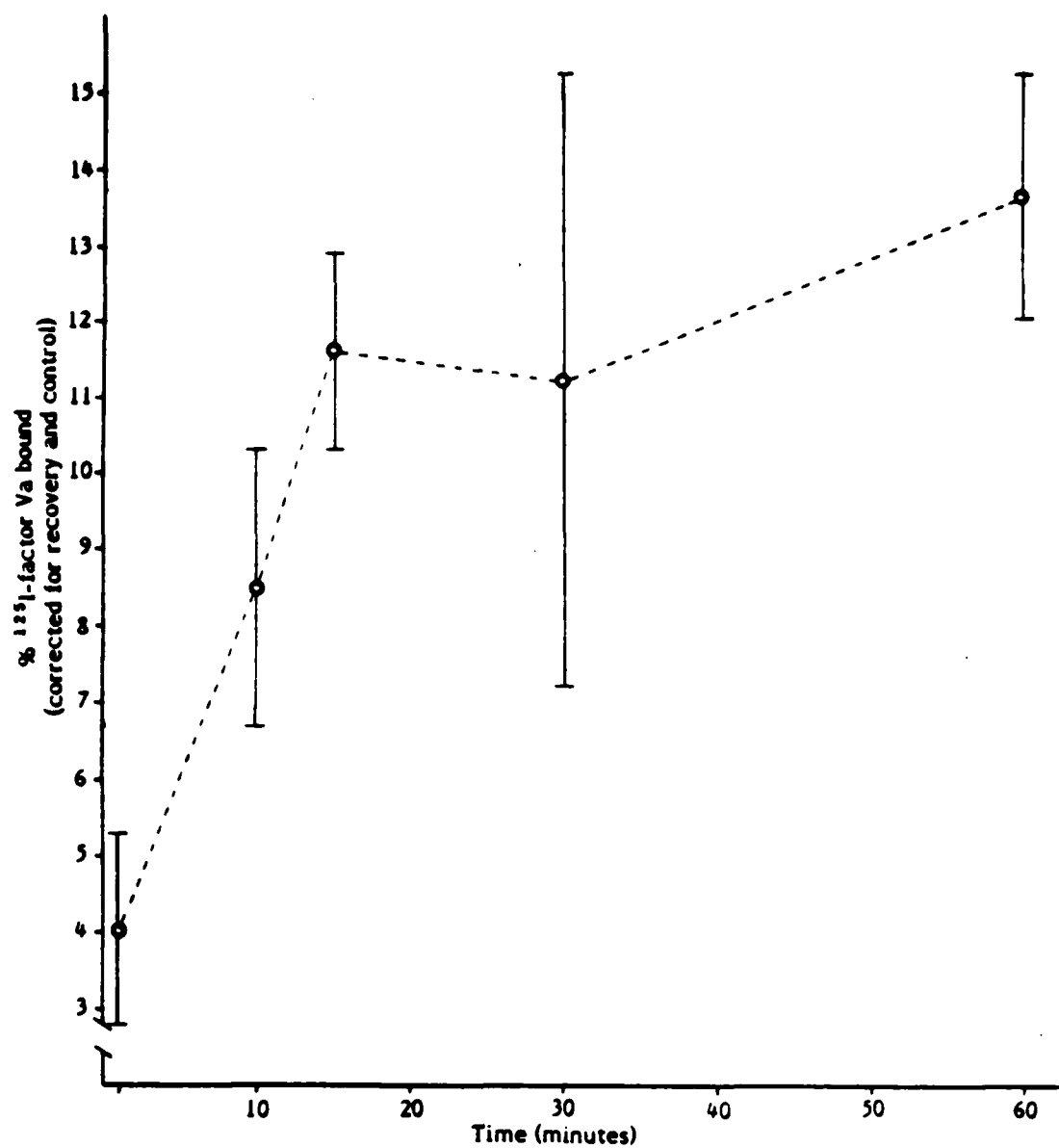


Figure 18



Figure 18. Factor Va binding to endothelial cells on Cytodex 3 microcarriers. Bovine aortic endothelial cells were cultured on cytodex 3 microcarriers in multi-well culture dishes until confluent. Cells, on the microcarriers, as well as control no-cell microcarriers, were gently washed two times with Tyrodes-albumin-5 mM HEPES, pH 7.4. Activated  $^{125}\text{I}$ -factor V was added to the reaction mixture at 50 ng/mL. The reaction mixture was made 30 uM DAPA to inhibit further thrombin action. Samples were taken, centrifuged through apiezon oil-butyl phthalate, and the cell-bead pellet was counted in a gamma counter. Data was corrected for recovery and control prior to plotting, data points and error bars represent the mean and standard deviation of duplicate samples.

settling of the beads damaged cells adherent to them, causing "blebs" and fragments to be released into the supernatant reaction mixture, arose when the supernatant was removed, assayed, and it was noticed that the rate of change in fluorescent signal remained the same as when cells were present. To further pursue this "blebbing" theory, BAE cells on microcarriers were processed for scanning electron microscopy, both before, and after, the DAPA assay reaction. Photomicrographs are as shown in Figures 19 and 20. The close up of one cell, shown in Figure 20, reveals small polyp-like blebs on the cell's surface. In an attempt to isolate these blebs, assuming they were dispersed into the reaction mixture, centrifugation in a Brinkman-Eppendorf microfuge was accomplished, but the supernatant retained activity. Thus, cells grown on microcarriers were abandoned for the DAPA assays, and cells grown in cuvettes were used.

The number of cells at confluence averaged  $4.5 \times 10^5$  in the  $1 \text{ cm}^2$  cuvette, as determined by cell counting in a hemacytometer, following trypsin-EDTA dissociation of the cells from a control cuvette. The cells in the cuvettes were carefully rinsed with reaction buffer, immediately prior to use. Again, the amount of prothrombin generated was not significantly greater than the no cell control cuvette. As seen in Figure 21, the cells provided only 2-3 times the amount of prothrombin produced, as opposed to the expected order-of-magnitude increase predicted, if the cells were providing a useable prothrombinase receptor, as do platelets. It seemed apparent that BAE cells were providing a surface for prothrombinase formation; however, cell numbers three orders of magnitude less than the platelet studies done by Tracy et al.(51), in the same reaction volume, were providing thrombin production below the threshold of DAPA fluorescence detection capability. Therefore, a somewhat



Figure 19

Figure 19. Scanning electron micrograph of cytodex 3 microcarrier confluent with bovine aortic endethelium. BAE grown on cytodex 3 microcarriers retain their in vitro "cobble-stone" morphology. Cells prior to use in DAPA fluorescence assay. (Magnification 900x)

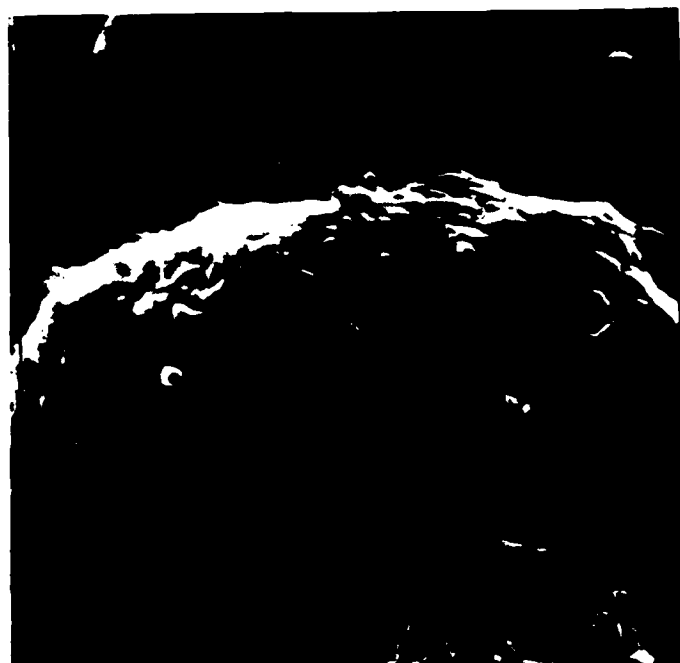


Figure 20

Figure 20. Scanning electron micrograph of bovine aortic endothelial cell growing on cytodex 3 microcarrier. After reactants were added for the DAPA assay, BAE cells were believed to be "blebbing", this higher magnification of an endothelial cell indicates polyp-like membrane structures, which may support this theory of blebbing. (Magnification 7000x)

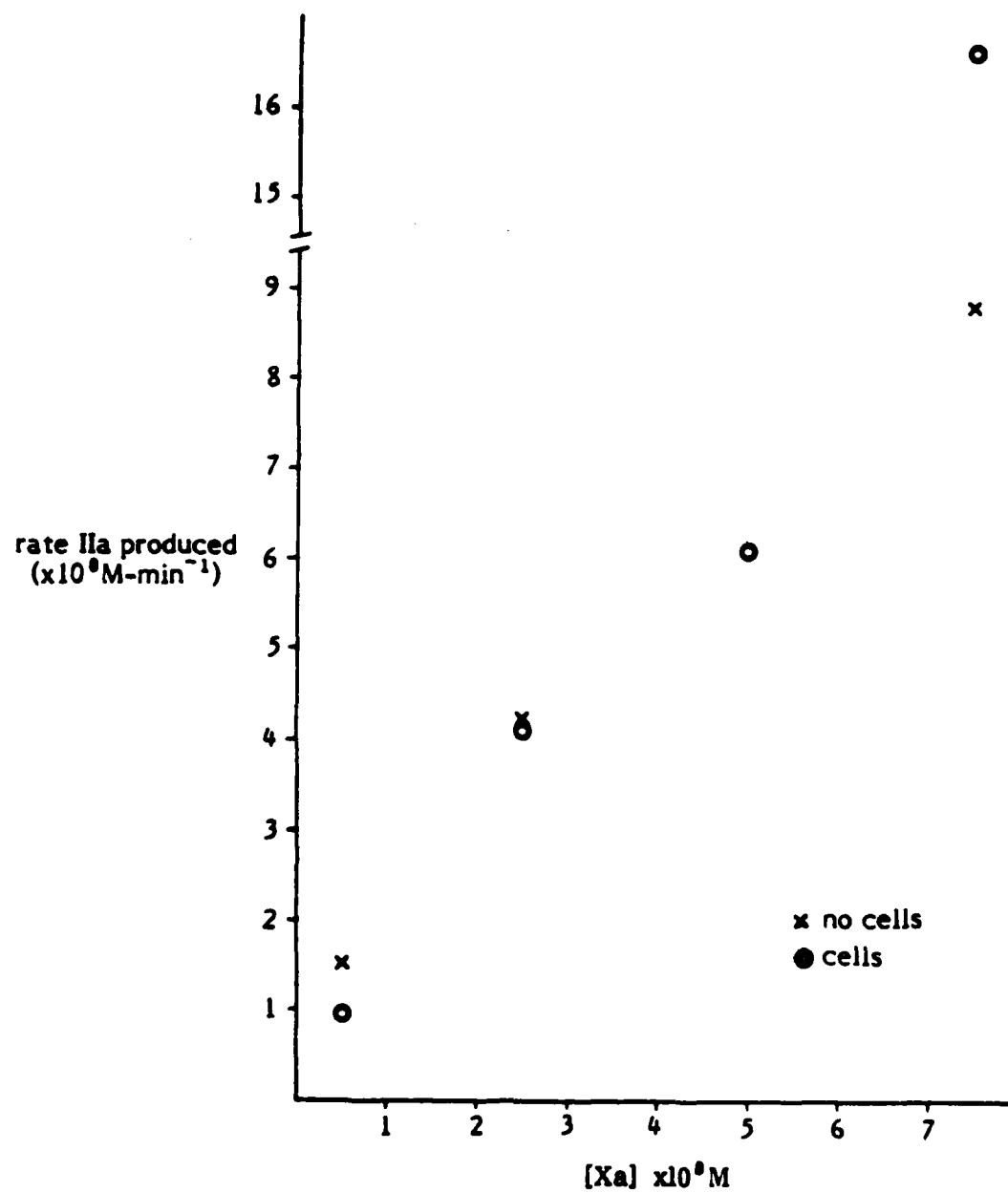


Figure 21

Figure 21. Thrombin production by BAE cells grown in cuvettes as determined by DAPA fluorescence. Cells grown on cytodex 3 microcarriers did not provide sufficient thrombin production to be detectable by the DAPA assay, therefore, cells were grown directly in the fluorimetry cuvettes. Confluent cuvettes were used in the assay, with control cuvettes containing no cells as a control. Cells provided levels of thrombin below the threshold of DAPA fluorescence detection capability and was virtually indistinguishable from the no cell control.



more sensitive chromogenic assay, using S-2238 as a substrate for thrombin, was used.

#### S-2238 Assays:

In the initial study, stimulation of cells was attempted using phorbol and thrombin as endothelial cell stimulants. As indicated in Figure 22, there was depression rather than stimulation by the two chosen substances. Since control cells provided such a positive indication of IIa production, attempts to stimulate cells was pursued no further. Attempted stimulation using thrombin seemed to inhibit thrombin production, as detected by the assay.

Follow on assays, as represented in figure 23, indicated that thrombin was produced at a significantly higher rate than in the C-3 microcarrier (no cell) control. In fact, the ninety minute time point indicated that endothelial cells provide a more efficient surface than the PCPS vesicle positive control, used as a standard for comparison.

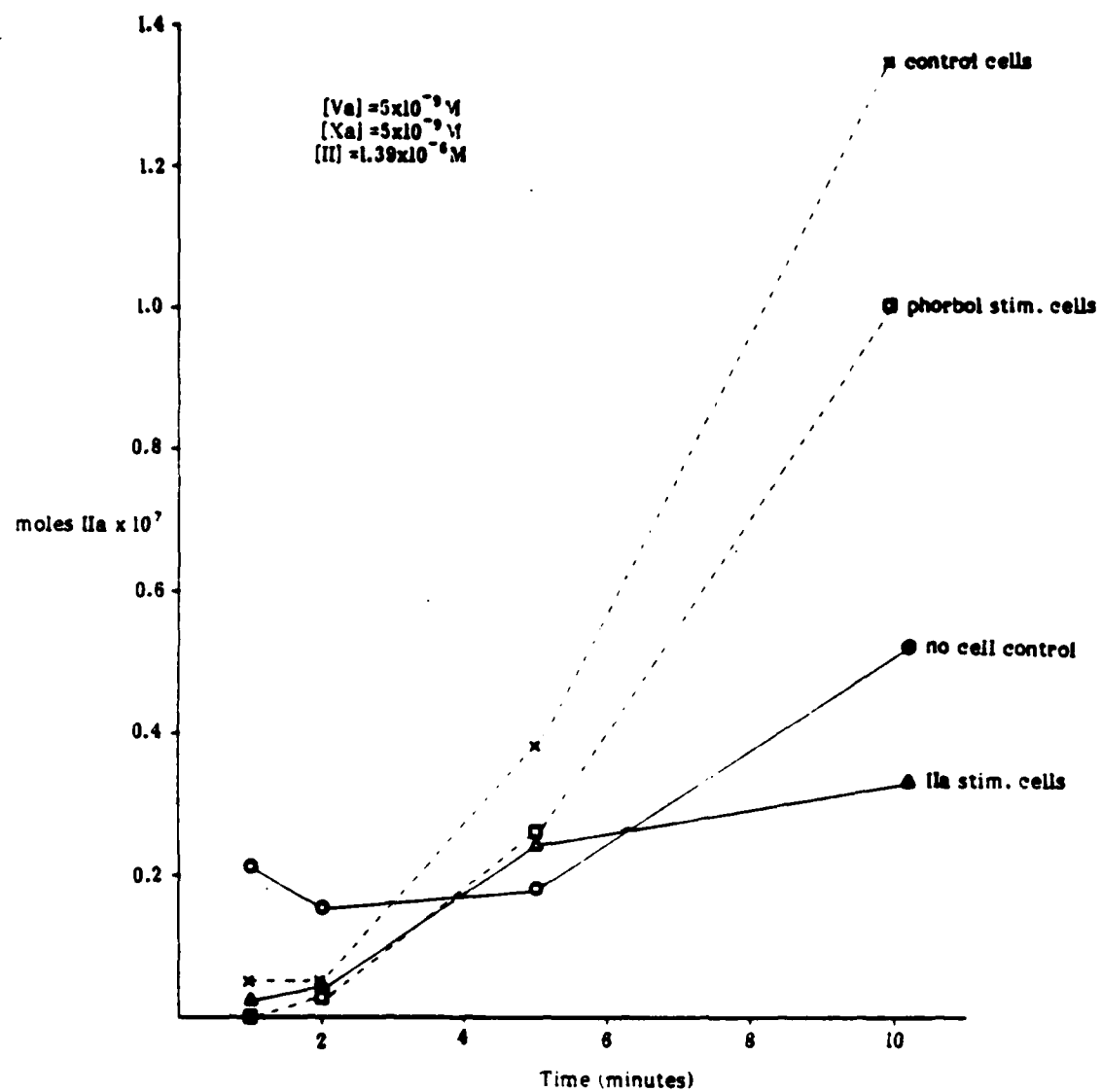


Figure 22

Figure 22. S-2238 assay of stimulated confluent bovine aortic endothelium grown in cuvettes. S-2238 provided a sensitive, chromogenic assay of thrombin production by cultured endothelium in cuvettes. Attempts to stimulate endothelial cells resulted only in an apparent suppression of endothelial ability to support the prothrombinase complex. Control untreated cells provided a positive indication of thrombin production, indicating that they provided a surface for prothrombinase formation.

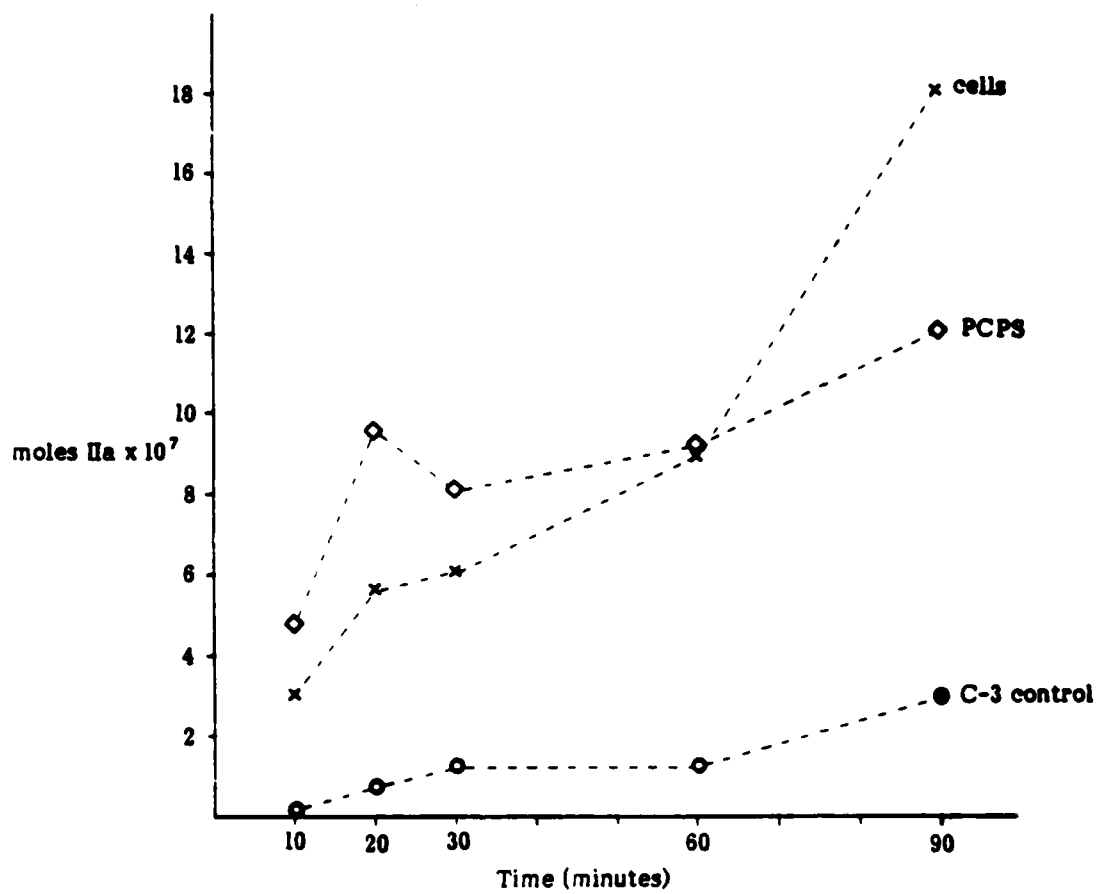


Figure 23

Figure 23. S-2238 assays of confluent bovine aortic endothelial cultures grown in cuvettes. Since thrombin production by cells in cuvettes was detectable by the S-2238 assay, a PCPS positive control was also accomplished for comparison. With time the cells appear to increase their production of thrombin, resulting in a curve similar to that of factor V binding (Figs. 17 and 18).

## DISCUSSION

Endothelial cells provide a continuous monolayer, lining the luminal surface of arteries, veins and lymphatic vessels, and are the only cell making up capillaries. There is evidence that endothelial cells vary in structure, and apparent function, throughout an organism, dependent upon the requirements of the tissue that it subserves (1,2). In addition to their obvious role in vascular permeability, endothelial cells synthesize a variety of biologically active products, and participate in blood clotting (1,2,12,13,21,53-61). Techniques for culturing endothelial cells, available only since the early 1970's (1,2), enable in vitro characterization of endothelial cell properties and products.

It has been demonstrated that endothelial cells synthesize and secrete a variety of proteins: fibronectin (56,57,60), collagen (53), and Factor VIII:vWF (21,54,62,63). Since endothelium was shown to produce factor VIII:Ag/vWF (12,13,21,54,62,63), its role in blood clotting has become more significant. The synthetic site of the other coagulation co-factor, factor V, has been as elusive as that of factor VIII.

Since bovine aorta was a readily available source of relatively large quantities of endothelial cells, it was the system of choice for study, though one experiment was accomplished using human umbilical vein endothelium. Bovine aortic endothelial cells, in vitro, synthesize and secrete three major proteins into the culture supernatant: fibronectin, a non-collagenous glycoprotein, and type III collagen (53). Fibronectin appears to constitute about 15% of the protein released into the culture supernatant (60). By comparison, one of the plasma coagulation factors, Factor VIII antigen, represents less than 1% of the

protein released into the culture supernatant (60). These figures are comparable to plasma levels of these two proteins. Since factor V levels in plasma are also much less than 1% of total plasma proteins, by analogy, in vitro cellular synthesis, and secretion of Factor V, were expected to be low. Human and bovine plasma levels of factor V are different. Human plasma levels of factor V range from 4-14 ug/ml, while bovine plasma levels of factor V range from 30-50 ug/ml (42). There are also differences in platelet factor V levels. Bovine platelets contribute about 2-5% of total bovine blood factor V, while human platelets provide 18-25% of total human blood factor V (42). Since platelets contain factor V, speculation as to whether or not their precursor, the bone marrow megakaryocyte, produces factor V has been hypothesized. The answer is currently under study in Dr. Mann's laboratory.

The two-stage bioassay provided preliminary evidence that, at least, Factor V-like activity could be found in culture supernatants, in excess of that present in unused, heat-inactivated FBS-supplemented medium. Thus, the theory that endothelium produces coagulation factor V became the quest of this thesis.

Several methods for detection/isolation of factor V were pre-existent: bioassay (32), radioimmunoassay (42,43), and immunoabsorption (29,36). In addition, autoradiography techniques were adapted to aid in identifying endothelial intrinsically-labelled factor V after recovery by immunoabsorption. Immunofluorescence techniques were developed/adapted for detecting antigen activity associated with endothelium in culture.

Bioassay provided immediate feedback concerning factor V presence in culture supernatant samples; however, solubilized cells and unused medium

revealed no detectable factor V bioactivity. The levels of bioactivity detected were low in comparison to plasma levels; however, these levels increased linearly once the culture became confluent. These increasing levels of factor V could be interpreted as endothelial cell synthesis of the coagulation cofactor. The lack of detection of factor Va in the first stage of the bioassay gave reason to speculate that, either factor V was not being proteolyzed, or that "synthesized" factor V was being processed, by some mechanism, to non-bioactive subunits, under the given culture conditions. Positive results from bioassay studies were further confirmed by radioimmunoassay results.

The radioimmunoassay was more sensitive to fragments of factor V, due to the polyclonal nature of the antibody, whether those fragments were bioactive or not. Thus, results from radioimmunoassay of cultured supernatant indicated a greater amount of factor V antigen present than the bioassay had indicated factor V bioactivity. Solubilized BAE cells indicated levels of factor V antigenicity at the lower end of detectability of the radioimmunoassay. Solubilized human umbilical vein endothelial cells provided a higher level of antigenicity than 3-day culture medium; however, when medium was left for 2 extra days, factor V levels in the supernatant were greater than in solubilized cells. Perhaps human cells store factor V, and their bovine counterparts secrete it into the plasma. The aforementioned linearly increasing level of factor V activity/antigenicity in the culture supernatant was supportive, but not a conclusive indication, that the cultured endothelium had produced the factor V.

To further verify that endothelial cells had synthesized the increasing levels of factor V in culture supernatants, confluent cultures were dosed with



<sup>35</sup>S-methionine in methionine-free medium. The cultured endothelium was allowed to uptake and incorporate the <sup>35</sup>S-methionine into proteins being synthesized. As mentioned earlier, factor V was much less than 1% of the proteins being synthesized; therefore, cultures were allowed to uptake and use the labelled methionine for four to five days, dependent upon objectively determined changes in culture morphology, before harvesting the culture supernatant for immunoisolation procedures. Bovine cells had provided such low levels of factor V activity/antigenicity, that, they were discarded. Immunoisolation was monitored by bioassay and beta scintillation counting of fractions throughout the various washes. The elution step using glycine at pH 2.8 eliminated bioactivity, thus, the fractions were followed exclusively by scintillation counting. Considering the radioactivity initially retained by the antibody-Sepharose, the peak fractions eluted were a small portion of that which had been applied; however, as previously mentioned, this was anticipated.

Initial autoradiography attempts resulted in such heavy background that factor V was impossible to identify. To clean up the background, intermediate high salt (1M and 2M NaCl) and 1% triton washes, shown by Foster et al. (36) not to dissociate factor V from the immunoabsorbant, were used to decrease background from the eluted materials. These washes removed some of the lower molecular weight materials, thereby decreasing background, such that more of the beta counts applied to the acrylamide gel were potentially factor V. The autoradiograph in Figure 16, still shows some low molecular weight background materials; but the higher molecular weight bands are in line with the factor V/Va iodinated standards. Thus, the highly specific immunoisolation of intrinsically labelled cellular synthetic products had provided an immuno

isolate that co-migrated with the Bolton-Hunter  $^{125}\text{I}$ -labelled factor V standards. In addition, the immunoisolated  $^{35}\text{S}$ -labelled protein had also been proteolytically cleaved by bovine thrombin, yielding fragments which co-migrated with thrombin-cleaved  $^{125}\text{I}$ -factor Va standards. Now, there was conclusive evidence that cultured bovine aortic endothelial cells had synthesized factor V. This evidence was further supported by the fact, that the factor V produced could be proteolytically cleaved, and result in fragments electrophoretically co-migrating with factor Va.

Immunofluorescence was employed to further determine whether the substance produced by the cultured endothelium was factor V. Primary and passaged cultures which were preconfluent, confluent and varying days post-confluent were used initially, until results indicated that 2-3 days post-confluent was the optimum time for immunofluorescence detection. The fluorescence of factor V was noted to be very fine, granular, and diffuse over the cytoplasm of fluorescing cells. Nuclei did not fluoresce. Indeed, not all cells exhibited fluorescence; it was as though a cell had to be in a particular stage of its cell cycle, or in a specific metabolic state, to exhibit the antigen. Monoclonal and polyclonal antibodies both resulted in positive fluorescence; however, there was much greater contrast (intensity) with the polyclonal antibody. The monoclonal data was significant in providing insight into antibody specificity, even though the intensity was weak. The polyclonal von Willebrand factor IgG resulted in fluorescence of bright granules over the entire cell, often with a fluorescent granular matrix surrounding the cells. This immunofluorescence is a characteristic typical of endothelial cells (20,49,50).

Bioassay, radioimmunoassay, immunofluorescence and, most importantly, immunoisolation data demonstrate that cultured bovine aortic endothelial cells synthesize factor V, and, at least under the in vitro culture system used, endothelial cells provided factor V as a secreted protein product. Results from immunofluorescence indicate that regulation of factor V synthesis must exist, since all cells did not fluoresce. Whether the regulation is end-product controlled by feedback mechanisms, or secretagogue controlled, is to be elucidated. Since endothelium lines the luminal surface of the entire vascular tree, it provides a ubiquitous synthetic "factory", which may supply the plasma pool of factor V.

As previously mentioned, factor V is an essential cofactor in maintenance of hemostasis. It is one of the two proteins of the prothrombinase complex, the other is the enzyme factor Xa. The two proteins, especially factor Va, seem to be most significant in prothrombinase activity, though deletion of any one of the four prothrombinase components (factors Xa and Va, a phospholipid surface and calcium) decreases the reaction rate at least 1000 fold in conversion of prothrombin to thrombin. A factor V deficient patient mentioned by Tracy et al (42) has continuously required blood transfusions to maintain hemostasis. Therefore, the significance of factor V in hemostasis, and thrombosis, becomes very evident. Endothelium becomes very important in hemostasis and thrombosis with the finding that endothelial cells produce factor V. For many years, endothelium had been considered an "inert, non-thrombogenic lining of the vascular tree". Now that culture of endothelial cells is possible, the myriad of cellular synthetic products, being elucidated almost daily, indicates that endothelium plays a very significant metabolic role in maintaining blood flow,

providing energy substrates to underlying tissues, and, as discussed in this thesis, by producing coagulation factors, a significant role in hemostasis and thrombosis.

Recently, many researchers have suggested that thrombosis starts at the level of the vascular wall. Research data indicates that factor IX and factor IXa bind to confluent cultured bovine aortic and human umbilical vein endothelial cells (66). Further, that this binding was saturable, labelled ligand was inhibited by unlabelled ligand, and that binding was reversible. The observation that factor IXa, bound to the cell surface, retains its coagulant activity provides a mechanism for the localization of a potent clot-promoting activity to the vascular wall surface, which may be important in thrombosis and hemostasis. In another recent study, cultured bovine aortic endothelium, like platelets, possess receptors for factor Xa, and provide a surface for prothrombin activation. Prothrombinase activity requires factor V, which was found specifically associated with adult bovine aortic endothelium, furthermore, the reported increase in prothrombinase activity, associated with dividing endothelial cells, may be an important part of the response of endothelium to vascular injury (65). As determined by this thesis research, endothelial cells synthesize, and secrete, factor V. And, as mentioned earlier, factor V is postulated by Nesheim et al. (15) to be embedded in a phospholipid surface (such as a cell membrane) providing a large cleft, into which the enzyme factor Xa can be sequestered with prothrombin, in the presence of calcium, thereby greatly enhancing enzymatic action.

While determining the synthesis of factor V by endothelium, basic experiments to examine the proficiency of endothelial cells in providing a phospho-

lipid surface for prothrombinase complex formation were accomplished. Protocol, which had been established by Tracy et al. (51) in determining platelet interactions with prothrombinase, were modified for use with endothelial cells. Both techniques used measured thrombin production by adding prothrombinase components to cultured endothelium, which, if our hypothesis was correct, would provide the "phospholipid" source. The DAPA assay proved to have a sensitivity threshold below the capability of endothelial thrombin production. Therefore, a chromogenic assay, using S-2238, a thrombin substrate, which when cleaved provides a color change, was used with results as shown in Figures 22 and 23. The low levels of thrombin produced by endothelial cells, as compared to the DAPA assay platelet studies done by Tracy et al. (51), can be explained by cell numbers. The platelet studies used cell numbers three orders of magnitude greater than the endothelial cell experiments described here. In addition, the low level of prothrombinase activity may have been due to the cell cultures being confluent to post confluent. The information presented above (65) was accomplished on cell cultures that were preconfluent and still dividing, like the situation which may exist in trauma or damage to endothelium. It seems very logical that the cells would be able to provide factor V, as well as a surface, for the assembly of the prothrombinase complex in such a situation. In fact, the results from immunofluorescence may also substantiate this supposition, in that cells were differentially reactive to the factor V antibodies used in the technique.

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